STRUCTURAL AND BIOCHEMICAL ASPECTS OF POSTNATAL LUNG GROWTH IN EXPERIMENTAL DIABETES: THE ROLE OF ENDOGENOUS FACTORS

BY

ANWULI FELIX OFULUE

B.S. University of Wisconsin, 1976
M.Sc. University of Manitoba, 1980

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Department of Pathology

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date July 31, 1984
ABSTRACT

The effect of experimental diabetes on the lung was studied in male rats made diabetic by injecting streptozotocin at age 3 weeks and sacrificed at age 7 weeks. Since the diabetic animals showed poor weight gain, an undernourished control group of similar weight gain was instituted, giving the opportunity to simultaneously study the effect of undernourishment. The other groups studied were normal control animals with access to food ad libitum and diabetic animals treated with insulin.

The diabetic animals weighed 75% of the fed control but specific lung weight and lung volume were increased. These changes were associated with increased number and surface area of alveoli, decreased size of alveoli, increased collagen and elastin content although the rate of synthesis appeared normal, and decreased cell number but normal cell size. In contrast, the undernourished control animals had normal specific lung weight and lung volume associated with decreased alveolar number and surface area but increased size of alveoli, decreased content and synthesis of collagen and elastin, reduced cell number and cell size. The changes in the insulin-treated diabetic animals were midway between the values of normal control and untreated diabetic animals. In the lungs of all the experimental groups of animals, there was a positive relationship between the changes in connective tissue content and those in alveolar number; cellular proliferation was unrelated to alveolar number. Such finding supports the hypothesis that connective tissue
deposition rather than cellular proliferation is the major determinant of alveolar formation and maturation.

**In vitro** studies indicated that soluble lung extracts derived from the experimental animals had suppressive effects on DNA and connective tissue syntheses by normal lung mince in culture. The extracts of the undernourished control animals were more potent than those of the other experimental groups, and this appeared to be due to the increased activity of an adenylate cyclase cytosolic modulator present in the lung. The suppressive effect on connective tissue production by the extracts of the diabetic animals was less than those of the other animals, and this was apparently due to the reduced activity of a heat-stable 13700-17000 dalton protein probably associated with the ubiquitous Ca\(^{2+}\)-dependent regulatory protein known as calmodulin. The reduced cellular proliferation in the lungs of the diabetic animals was due mostly to a heat-stable 17000-25000 dalton protein which suppressed the ability of calmodulin to enhance DNA synthesis. The data indicate that changes in the activities of these endogenous factors mediated at least in part the structural changes observed in the lung in response to undernourishment and experimental diabetes.
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This thesis is dedicated to

Ifeoma Eni

my friend, my sister, my love
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GLOSSARY OF ABBREVIATIONS

DNA Deoxribonucleic acid

RNA Ribonucleic acid

DSPC Disaturated phosphatidylcholine

cyclic AMP Cyclic adenosine 3',5'-monophosphate

EGTA [Ethylene-bis(oxyethylenenitrile)]tetraacetic acid

PBS Phosphate-buffered saline

TCA Trichloroacetic acid

PCA Perchloric acid
I. INTRODUCTION

A. General Objective

The purpose of this investigation is to study the influence of streptozotocin-induced diabetes mellitus on the structural and biochemical aspects of postnatal lung growth and development in rats, and to determine the possible role of endogenous regulatory factors in the modulation of DNA, collagen and elastin syntheses in the lung. The relevance of the findings to human diabetes mellitus will be discussed.

B. Postnatal Lung Growth and Biochemical Development

Although extensive growth of the fetal lung occurs during gestation to prepare it for its role in gas exchange, at birth the lung is still not fully mature in most mammalian species (1). The degree of structural maturity of the mammalian lung is associated with the number of alveoli and the alveolar surface area present in the organ. Morphometric studies of human lungs (2-4) demonstrated that there are about 20-50 million alveoli at birth compared to 200-600 million alveoli present in adult life. The average alveolar size increases from 40-120 μm at 2 months of age to 250-300 μm in the adult. There is a 12-fold increase in alveolar surface area and a 28-fold increase in lung volume from birth to adult life. The recent study of postnatal human lung growth by Thurlbeck (4) indicated that the size of alveoli and the number of alveoli per unit volume did not change significantly during the first 2 or 3 years of life. After this age, the alveolar size steadily increased with a
concomitant decrease in the number of alveoli per unit volume. Rapid 
alveolar multiplication predominated during the first 2 years of life 
whereas the enlargement of alveoli characterized the growth thereafter. 
It is thus apparent that postnatal growth of the human lung is 
characterized by increase in number and size of alveoli.

In newborn rats and mice, definitive alveoli are absent. The 
blood-gas barrier is present as tubular, relatively smooth-walled 
structures referred to as "primary saccules" and the total surface area 
for gas exchange is small. Postnatal events establish the large gas 
exchange surface area characteristic of the mature adult lung (1). 
Morphologic studies (5,6) indicate that soon after birth, the saccules 
become irregularly contoured by a large number of humps of tissue 
protruding into the air spaces. These newly formed tissue septa 
subdivide the saccules into definitive alveoli and alveolar ducts. Based 
on quantitative morphometric findings (see Table I and Figure 1) and 
autoradiographic data, Burri and Weibel (6) described three distinct 
phases of postnatal lung growth in rats. The first phase, which lasted 
from birth to day 4 of postnatal life, was described as that of lung 
expansion. During this phase, lung volume increased less than body 
weight such that the ratio of lung volume to body weight (the specific 
lung volume) was decreased. The lung volume change was particularly 
associated with enlargement of the primary saccules, with no apparent 
change in volumes of the tissue compartments. The second phase was 
described as that of rapid tissue proliferation and lasted from day 4 to
Table I: Synopsis of Quantitative Findings in Postnatal Growth of Rat Lung (6).

<table>
<thead>
<tr>
<th>Age in days</th>
<th>BW (g)</th>
<th>VL (cm³)</th>
<th>Vₙ (cm³)</th>
<th>Vₛ (cm³)</th>
<th>Vₕ (cm³)</th>
<th>Vₑ (cm³)</th>
</tr>
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<td>1</td>
<td>7.2±0.1</td>
<td>0.57±0.01</td>
<td>0.302±0.008</td>
<td>0.177±0.012</td>
<td>0.135±0.012</td>
<td>0.042±0.001</td>
</tr>
<tr>
<td>4</td>
<td>12.0±0.2</td>
<td>0.90±0.03</td>
<td>0.565±0.013</td>
<td>0.187±0.015</td>
<td>0.145±0.001</td>
<td>0.042±0.014</td>
</tr>
<tr>
<td>7</td>
<td>15.6±0.2</td>
<td>1.22±0.05</td>
<td>0.690±0.013</td>
<td>0.317±0.036</td>
<td>0.251±0.024</td>
<td>0.066±0.014</td>
</tr>
<tr>
<td>10</td>
<td>18.5±0.3</td>
<td>1.44±0.07</td>
<td>0.814±0.019</td>
<td>0.365±0.036</td>
<td>0.289±0.034</td>
<td>0.076±0.005</td>
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<td>13</td>
<td>25.7±0.7</td>
<td>1.80±0.06</td>
<td>1.035±0.068</td>
<td>0.512±0.022</td>
<td>0.377±0.023</td>
<td>0.135±0.011</td>
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<td>21</td>
<td>38.5±0.1</td>
<td>2.25±0.11</td>
<td>1.311±0.173</td>
<td>0.551±0.051</td>
<td>0.354±0.031</td>
<td>0.197±0.030</td>
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<tr>
<td>44</td>
<td>140.5±7.1</td>
<td>6.34±0.20</td>
<td>4.143±0.108</td>
<td>1.047±0.060</td>
<td>0.565±0.040</td>
<td>0.480±0.022</td>
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<td>131</td>
<td>456.7±12.0</td>
<td>13.37±0.52</td>
<td>8.130±0.337</td>
<td>2.398±0.056</td>
<td>0.919±0.041</td>
<td>1.479±0.025</td>
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BW = body weight; VL = lung volume; Vₙ = alveolar volume (alveoli and alveolar ducts); Vₛ = septal volume (= Vₕ + Vₑ); Vₕ = parenchymal tissue volume; Vₑ = capillary volume.
Figure 1: Alveolar surface area ($S_a$) plotted bilogarithmically against lung volume ($V_L$) (6).

Between days 4 and 21, the slope is very steep; it corresponds to the period of formation and deepening of the alveoli.

After day 21, the slopes are reduced but more alveoli are added.
13 of postnatal life. During this phase, lung volume increased faster than body weight such that specific lung volume actually increased. All tissue compartments showed pronounced increases in volume density, and alveolar surface area increased to the 1.6 power of lung volume, a rate compatible with formation of new alveoli. Active proliferation of fibroblasts, endothelial and epithelial cells of the peripheral lung tissue occurred during this phase. The third phase was described as that of "proportionate" growth and began at approximately 2 weeks of age. Body weight increased faster than lung volume and thus the specific lung volume decreased. This phase was further characterized by enlargement of epithelial cells of the peripheral lung tissue and septal lengthening. Alveolar surface area increased to the 0.71 power of lung volume, very close to the 0.67 power that would come by simple enlargement of the existing alveoli. However, based on the morphometric increase in septal and parenchymal tissue volumes after the third week of postnatal life, it was suggested that further alveolar multiplication may be present during this phase of lung growth.

The quantitative morphometric data of Holmes and Thurlbeck (7; see Figure 2) indicated that alveolar multiplication in rat lungs occurred between 4 and 10 weeks of postnatal life while surface area increased directly with lung volume from 4 to 14 weeks. There were indications that the increase in lung volume was accompanied by some increase in alveolar dimension. Thus it appears that the phase of "proportionate" growth described by Burri and Weibel (6) may be that of continuing but
Figure 2: Normal lung growth of the rat; morphometric variables plotted against age (7). Lung volume ($V_L$), lung weight, and surface area (SA) increased significantly with age. Number of alveoli per unit volume ($N_v$) and average alveolar transection length ($\bar{l}_{alv}$) did not change significantly with age, but did indicate a trend toward larger alveoli in older animals. The total number of alveoli ($N_{AT}$) increased dramatically between 4 and 10 weeks of age.
slower alveolar multiplication accompanied by some enlargement of existing alveoli. The available data (1,5,7) have suggested a fourth phase of postnatal lung growth, characterized by simple lengthening of all the existing alveolar walls, which would start when alveolar multiplication ceased. This phase appeared to start at about 10 to 14 weeks of postnatal life in rats.

There are no doubts that alveolar formation is accomplished by septation of the primary saccules. The ends of the growing septa have elastin and collagen fibres, and the hypothesis to date is that the septal network of thick elastic tissue fibres may function as a mold through which the lung parenchyma protrudes to form the alveolar outpouchings (1,5,6). Vaccaro and Brody (8,9) have described two types of fibroblasts that produced both collagen and elastin in the developing alveolar structures in rat lungs. One type appeared within the alveolar buds and was involved in the deposition of microfibrillar and amorphous components of elastin in association with the formation of new alveoli. The other type of fibroblast was in the alveolar walls and was characterized by an accumulation of lipid droplets. Although this cell appeared to be less closely associated with connective tissue production, it was evident in greatest number and contained the most lipid during the period of rapid alveolar formation. The lipid in this cell then disappeared as alveolar formation ceased. Although the function of the lipid within this cell remained unclear, it was interesting that the lipid-filled interstitial cell also contained actin-like filaments which
often traversed the alveolar wall, indenting the epithelial basement membranes, and apparently serving as anchor points for the growing lung structures (9). However, morphologic studies of postnatal lung growth in humans and in animals (1,5,10-12) suggest that alveoli may form by segmentation of already existing alveoli and also directly from respiratory bronchioles and alveolar ducts. In addition, transformation of one or two terminal bronchioles into respiratory bronchioles may occur as a result of formation of alveoli, a process called alveolarization of bronchioles.

Biochemical measurements of postnatal lung growth in rats by Winick and Noble (13) demonstrated that growth proceeded initially mainly by cell division (as indicated by increasing amounts of DNA) and then by cell enlargement (as indicated by the increase in weight and in amounts of protein and RNA relative to those of DNA). The increase in RNA throughout the growth period was not always proportional to that of DNA, resulting in changes in the RNA:DNA ratios. This suggested that there was alteration in the amount of RNA present to sustain cellular protein synthesis during postnatal lung growth and development. Several investigators (1,13-15) have indicated that these cellular parameters provide the biochemical correlates of the structural events that characterize the postnatal lung growth processes. However, the lung produces a variety of chemical substances, and maturation of this synthetic capability is also linked with the structural aspects of lung growth and development. Two of these substances, pulmonary surfactant
and connective tissue macromolecules, particularly provide insight into the interaction between the structural integrity of postnatal lung growth and its biochemical development (16).

Pulmonary surfactant, a surface-active material, produced and secreted by alveolar epithelial type II cells, forms an acellular monolayer that lines the alveolar surface and lowers the surface tension at the air-liquid interphase, thus preventing alveolar collapse during expiration and reducing the inspirational force required to expand the lungs (17). Chemical analysis has shown pulmonary surfactant to be a mixture of phospholipids and small amounts of proteins and carbohydrates. Disaturated phosphatidylcholine (DSPC), the major phospholipid and surface-active component, is frequently used as a marker for pulmonary surfactant (18-21). The amount of pulmonary surfactant forming a monolayer film over the alveolar surface may reflect the gas exchange surface area of the lung (22). Studies of changes in phospholipid content of rat lungs during postnatal development correspond with the observed growth phases (15,23). Thus the measurements of lung phospholipid, particularly the airway DSPC content, provide an additional biochemical assessment of the growth status of lung.

The most recently studied biochemical correlates of the structural events in postnatal lung growth are the connective tissue proteins, produced by mesenchymal cells (fibroblasts, contractile cells, smooth muscle cells), endothelial cells and probably by the alveolar epithelial
cells (24-29). As stated earlier, several morphologic studies in humans and experimental animals have associated extracellular connective tissue deposition, particularly of elastin, with alveolar formation. In animal studies, Kida and Thurlbeck (30,31) demonstrated that injection of growing rats with B-aminopropionitrile (BAPN), which inhibits lysyl oxidase, interfered with alveolar formation and maturation. Lysyl oxidase, a copper-dependent enzyme, is responsible for the crosslinking and maturation of collagen and of elastin. Blotchy mice and copper-deficient rats, both of which are unable to form mature crosslinked connective tissue proteins, have few but large alveoli in their lungs (32,33) similar to those in the BAPN experiment. The high activity of lysyl oxidase in parenchymal tissue of rabbit lungs during the first 3 weeks of postnatal life decreased by fifty percent to a stable level by 4-10 weeks (34). While all these studies (30-34) in animals support a causal link between connective tissue maturation and alveolar formation, they do not define clearly the specific roles of elastin and/or collagen maturation. The only available biochemical study of lung elastin development in humans showed a rapid postnatal increase in elastin content which reached the adult level of 12% of lung dry weight by 6 months of age (27). The biochemical data from animal studies (26,28) agree with the morphologic observations in humans that collagen-fibril formation is less important than that of elastin in alveolar formation, since the collagen content increased only 3-fold between days 2 and 20 of postnatal life in rats whereas elastin increased 10-fold in the same interval that corresponds closely with the appearance
of alveoli. Elastin synthesis, measured by $[^3H]$valine incorporation into an elastin fraction of lung, occurred predominantly during the period of septation of the primary saccules into alveolar ducts and alveolar sacs (29).

Although elastin formation and maturation may be the key connective tissue component involved in the structural events of postnatal lung growth, recent morphologic studies by Brody and Vaccaro (35,36) have noted changes in alveolar epithelial basement membrane proteoglycans and capillary endothelial basement membrane continuity which suggest that these structures may play a significant role in the organization of developing alveolar walls. The link between collagen deposition and alveolar formation remains undefined by biochemical methods of measurement. Pickrell (37) has suggested that since collagen is a structural protein, its reduced synthetic rate in the presence of a constant rate of breakdown with increasing age in hamster, rat and rabbit lungs may reflect a decreasing instantaneous growth rate of the lungs. Collins (38) recently observed that the total collagen content of the large airways and blood vessels and of the peripheral parenchymal tissue increased approximately 12- and 90- fold, respectively, between 1 week and 6 months of postnatal life in the rabbit lung. However, the collagen content per gram of tissue in the large airways and blood vessels was 3-5 times that in the peripheral airways. Brody et al (34) have found that the lysyl oxidase activity of large airways decreased more slowly than that of the lung peripheral tissue during postnatal lung development in
the rabbit, remaining high for the first 10 weeks of age, and therefore suggested active synthesis of the airway connective tissue over a longer period of time than in the lung parenchyma.

Functional studies have made apparent the changes in lung mechanics that accompany postnatal lung growth and organization of connective tissue proteins within the lung (26,39-41). The extracellular matrix of the alveolar septa has been described as an important determinant of lung mechanics and structural stability (25,41). The volume-pressure relationship of the lung can be separated into two aspects:- one at low and mid lung volumes where elastin determines recoil, and another at high lung volumes where collagen limits lung distensibility. Nardell and Brody (26) recently explored the mechanical consequences of the structural events associated with postnatal lung growth in the rat. Their data indicated that the major increase in lung elastin per gram of tissue (noted earlier to correspond with alveolar formation) was associated with an increase in lung elastic recoil; when the elastin content plateaued, so did recoil. In contrast, lung collagen content correlated more closely with the postnatal increase in the distending pressure which the growing lung can sustain. Changes in lung volume (at 10 cm H_2O pressure) corrected for lung weight closely followed the elastin rather than the collagen content.

The patterns of cellular growth and extracellular deposition of pulmonary surfactant and connective tissue elements during postnatal lung
growth suggest that an effective regulatory system controlling these processes must be present in the lung. Of particular interest in this regard is cyclic AMP, a chemical substance produced in mammalian tissues by the action of a membrane-bound enzyme, adenylate cyclase, and degraded by the action of cyclic AMP-phosphodiesterase. Cyclic AMP has been ascribed the fundamental regulatory function of maintaining the differentiated state in tissues (42). Nijjar (23) demonstrated that lung cyclic AMP levels during postnatal development in rats closely approximated the growth phases, with a reduced level occurring during the phase of rapid tissue proliferation. The alterations in cyclic AMP levels resulted from changes in the activities of adenylate cyclase and cyclic AMP-phosphodiesterase. Cyclic AMP has been shown to modulate the growth of lung fibroblasts and their production of collagen \textit{in vitro}, with DNA and collagen syntheses inversely related to the cyclic AMP content of the cells (43,44). These findings (23,43,44) thus suggest that cyclic AMP may play a regulatory role in postnatal lung growth and development. Moreover, cyclic AMP stimulated the secretion of pulmonary surfactant phospholipids by alveolar epithelial type II cells \textit{in vitro} (45,46) and thus may be significant in determining the amount of surfactant required at the alveolar surface during postnatal lung growth.

C. Cyclic AMP Modulatory Systems in Postnatal Lung Development

Several studies (47-49) have suggested that the activity of adenylate cyclase and thus the formation of cyclic AMP in the developing rat lungs may be hormone-dependent, based on the well-established
findings that most hormones bind to specific receptors on the surface of their target cells and thereby cause the stimulation of adenylate cyclase. The variability in enzyme activity during postnatal lung growth and development may thus be due to changes in hormone levels and/or differential sensitivity of the enzyme to the same hormone as a result of variable concentration of hormone receptors on the target cells. However, Nijjar (50,51) and Whitsett et al (52) have shown that even the most implicated of the hormones, the B-adrenergic catecholamines, enhanced only late fetal and early neonatal rat lung adenylate cyclase activity with little or no effect on the enzyme of adult lung and suggested that this observation may be related to the role of cyclic AMP in the synthesis and secretion of pulmonary surfactant. Tordet et al (53) have demonstrated that changes in lung catecholamines and the cyclic nucleotides during perinatal development correlated well with the biochemical and morphologic maturation of the rat lung. Thus the early neonatal rise in lung adenylate cyclase activity and the level of cyclic AMP appears to be modulated by hormones.

There was a second rise in the cyclic AMP levels of developing rat lungs after day 15 of postnatal life, and Nijjar (50) has indicated that this may be related to the appearance of a cytosolic activator of the particulate adenylate cyclase in the rat lungs. The cytosolic factors have been characterized and shown to consist of two synergistic heat-labile proteins of 14500 and 65000 daltons (54). The kinetics of adenylate cyclase activation revealed that both the $K_m$ and $V_{max}$ were
affected and that there was a lag phase in the activation of adenylate cyclase by the cytosolic factors, suggesting an interaction between the cytosolic factors and components of the adenylate cyclase system. This cytosolic modulation of the particulate adenylate cyclase activity in the rat lungs appeared at a time when the rate of cellular and tissue proliferation was reduced, and hence it has been suggested that the cytosolic modulation of adenylate cyclase may constitute an important regulatory mechanism of the events associated with postnatal lung growth and development (7,16,23,50-52,54).

A ubiquitous heat-stable, 17000 dalton, calcium-dependent protein, referred to as calmodulin, modulates the activities of adenylate cyclase (in some mammalian tissues) and cyclic AMP-phosphodiesterase as well as the actions of cyclic AMP. In addition, it plays a pivotal role in cellular regulation since it modulates glycogen metabolism, phospholipid synthesis, secretory processes and a variety of other cellular events particularly cellular proliferation (55-57; see Figure 3). Ofulue and Nijjar (58) and Sharma and Wirch (59) have demonstrated the presence of calmodulin in rat and rabbit lungs, respectively, and its ability to modulate the lung cyclic AMP-related enzymes. According to the data reported by Ofulue and Nijjar (58), exogenous calmodulin activated adult lung particulate adenylate cyclase only when the particulate fractions containing the adenylate cyclase have been extensively washed in EGTA prior to assay to deplete them of excess endogenous calcium and calmodulin. The activation to normal basal level was calcium-dependent,
Figure 3: The functions of calmodulin in cellular regulation.
immediate, reversible and due to an increase in $V_{\text{max}}$ without any apparent effect on the $K_m$ values for the enzymes. These findings suggested that lung particulate fractions contained an adenylate cyclase that required calmodulin for the expression of its basal activity. The effects of the cytosolic factors and of calmodulin on the lung particulate adenylate cyclase were additive and apparently acted by different mechanisms.

Ofulue and Thurlbeck (60; see Figure 4) have recently studied the changes in lung calmodulin levels and the adenylate cyclase activity during postnatal development in rats. The data indicated that the calmodulin content increased to high levels by day 7 of postnatal life, remained so till day 21 and decreased thereafter. Calmodulin activation of lung particulate adenylate cyclase was prominent at day 3 of postnatal life but gradually decreased to little or no effect by day 14 when the influence of the cytosolic factor began to appear. In these studies, the lung particulate fractions were not treated with EGTA and thus likely represented the physiological condition in vivo. High level of calmodulin was apparent during the phase of rapid tissue proliferation in the rat lungs, and calmodulin has been implicated in the growth of other mammalian tissues (61-63). However, the growth-related changes in the activity of the lung adenylate cyclase cytosolic factor followed closely the change in connective tissue content. Although the significance of these findings is not clear, the data suggest that the modulation of adenylate cyclase by calmodulin and by the cytosolic factor may play independent roles during postnatal lung development.
Figure 4a: Calmodulin and adenylate cyclase activity in lung homogenates (A) and particulate fractions (B) of the rat lung during postnatal development (60).
Adenylate cyclase activity (pmole cAMP/min/mg protein)

Calmodulin content (μg/mg protein)
Figure 4b: Effects of calmodulin (A) and calmodulin-depleted lung cytosol (B) on particulate adenylate cyclase activity of the growing rat lungs (60).
A. Calmodulin

Adenylate cyclase activity

% of control

300

200

100

3 7 14 21 42

Age (days)

5 µg

10 µg

B. Calmodulin-depleted Lung Cytosol

10 µg

5 µg

100
D. Alterations of Postnatal Lung Growth

Holmes and Thurlbeck (7) and other investigators (64-68) have demonstrated in animal studies that compensatory growth occurred in the lung when portions of the lung were collapsed or removed. This adaptive growth response resulted in increased weight, volume and alveolar surface area of the remaining lung. Studies of unilateral pneumonectomy have further characterized this growth response by observations of enhanced production of DNA, RNA, protein, and collagen, with hyperplasia of alveolar epithelial, interstitial, endothelial and pleural cells (67,68) and formation of new alveoli (7,66,69). A reduction in the lung cyclic AMP level (70) occurred during the period of maximal post-pneumonectomy growth response. However, the compensatory growth response after pneumonectomy appeared incomplete since the contralateral lung did not match both lungs of the control animals with regards to the structural growth parameters, except for weight (7,71). Various stimuli, including mechanical stretching of the lung by the thoracic cage and increased blood flow which removed endogenous inhibitors of cellular proliferation, have been suggested for the post-pneumonectomy growth response (1,16).

Smith et al (72) have demonstrated that serum from pneumonectomized rats stimulated DNA synthesis by alveolar epithelial type II cells \textit{in vitro}. There were no effects on lung fibroblasts. Partial characterization of the post-pneumonectomy serum suggested that the mitogenic factor may be somatomedin, a growth hormone-related mediator.

Brody et al (73) observed that postnatal lung growth may be altered
by changes in the level of growth hormone, since excess growth hormone in acromegalic men was associated with large lungs while deficiency of growth hormone resulted in small lungs with fewer alveoli. The influence of growth hormone on lung growth has been demonstrated in immature and adult animals (14), and the increased lung size in the presence of excess growth hormone was believed to be due mainly to hypertrophy of structural elements and probably some cellular hyperplasia. Brody and Buhain (74) indicated that growth hormone deficit suppressed post-pneumonectomy compensatory growth. Moreover, the effects of excess growth hormone and pneumonectomy on postnatal lung growth were additive and evidently acted by different mechanisms.

Cunningham et al (75) have demonstrated that postnatal growth of the lung structurally adapted to hypoxic conditions, with increase in the number and size of alveoli and alveolar ducts in young rats but increase in size and not in number of the structures in adult rats. These findings were supported by the fact that early post-operative exposure of animals to hypoxia accentuated the lung growth response following pneumonectomy (76). The mechanism of this growth response is unclear, although Brody et al (34) have shown a rapid 2-3 fold increase in lung lysyl oxidase activity and suggested that increased connective tissue synthesis was probably involved. As regards the effect of hyperoxic conditions, Bartlett (77) has indicated that hyperoxic exposure of growing rats resulted in smaller lungs with fewer alveoli and diminished surface area for gas exchange.
Burri and Weibel (6) have suggested that lung structure may be adapted to oxygen consumption, with an augmentation in the gas-exchange surface area reflected by larger numbers of small alveoli when oxygen consumption is increased. This hypothesis was based on the findings that rats vigorously exercised by swimming showed an increased number of alveoli in their lungs and that the Japanese waltzing mice which have a congenital defect of the vestibular apparatus and brain that subjected them to continued rapid movement and thus 80% higher oxygen consumption than the conventional white mice, have increased specific lung volumes and specific alveolar surface areas. The greater change in alveolar surface area than lung volume in the Japanese waltzing mice indicated that these animals had finer subdivisions (more alveoli) per unit of lung volume. However, Bartlett and Areson (78) found no change in lung growth in rats exercised daily on a treadmill for 20 days.

Brody and Vaccaro (9) have documented that postnatal lung growth may be adaptable to the nutritional status of the mammal, since there was inhibition of alveolar growth in lungs of growing hamsters deficient in dietary protein. Emphysema-like enlargement of air spaces and decrease in internal surface area (79,80) and reduced cell number (81), protein synthesis (82), lysyl oxidase activity (83) and amounts of elastin and collagen (84) have been indicated in the lungs of starved rats. However, D'Amours et al (85) have recently shown that starved rats have normal lung volume and alveolar surface area, although the amount of pulmonary surfactant present over the lung airways was reduced.
E. Diabetic Effects on the Postnatal Lung

Diabetes is a fundamental disorder of carbohydrate metabolism, with attendant alterations in lipid, protein and connective tissue metabolism, and has been described as "starvation in the midst of plenty" since its characteristic insulin deficit (absolute or relative) interferes with cellular utilization of glucose and glycogen storage producing hyperglycemia and glycosuria, the chief measurable aspects of the disease (86). Widespread abnormalities of connective tissue elements analogous to those which occur during aging, such as elastic fiber fraying (87), increased stiffness and nonenzymatic glycosylation of collagen and its resistance to enzymatic degradation (88), increased amount of cross-linked dimeric B-chains of collagen (89), and thickening of epithelial and capillary basement membranes (90) have been described in human and experimental diabetes mellitus. Altered levels of somatomedin and growth hormone (91-94) and hypoxemia (95-96) have also been indicated in the human disease. Thus it is apparent that general and systemic factors known to alter postnatal lung growth are present in diabetes mellitus.

Schuyler et al (97) suggested that postnatal lung growth and/or connective tissue maturation may be altered in diabetes. This hypothesis was based on the finding of loss of elastic recoil at low and middle lung volumes and of decreased total lung capacity in young men with insulin-dependent diabetes mellitus. However, the data for diabetic patients reported by Schernthaner et al (98) did not reveal any changes in lung elastic recoil and total lung capacity, and this may be due to
differences in the duration and age of onset of the disease in the patients studied. Balis et al (99) have indicated that neonatal respiratory distress syndrome, a characteristic abnormality in offsprings of diabetic mothers, retarded postnatal maturation of the premature lungs. Sugahara et al (96) observed that lung carbon monoxide diffusing capacity ($D_{LCO}$) was often lower in diabetic patients when compared to that in a corresponding age group. Changes in $D_{LCO}$ may be due to very diverse processes such as emphysema, fibrosis, ventilation-perfusion mismatching and the relative number of effective alveolar units (100). Madia et al (83) demonstrated that the lung content of acetic acid-soluble hydroxyproline and the activity of lung lysyl oxidase were increased in experimental diabetes. Kida et al (101) recently indicated by morphometric technique that induction of experimental diabetes in growing rats produced increased number of small-sized alveoli with attendant increase in the volume proportions of basal lamina, collagen and elastin in the alveolar walls of the lungs. It was suggested that the enhanced lung lysyl oxidase activity in experimental diabetes probably increased the cross-linking of the collagen-elastin network such that the individual links of the network became smaller and thus culminated in increased numbers of alveoli per unit volume.

Specific receptors for insulin have been identified in membrane preparations of normal rat lungs (102). Moreover, exogenous insulin therapy normalized the changes in lungs of short-term experimental diabetic animals, such as reduced glucose oxidation (102), suppressed
phospholipid and fatty acid synthesis (103,104) and dilatation of organelles in the alveolar epithelial type II cells and bronchiolar Clara cells (105,106). These findings suggested that insulin may be significant in the maintenance of the structural integrity of the postnatal lung. However, the effect of insulin therapy on long-term experimental diabetes-induced thickening of alveolar epithelial and capillary basement membranes (107) has not been determined. Insulin stimulated DNA synthesis by lung fibroblasts \textit{in vitro} (108). The role of cyclic AMP in the actions of insulin is well established in mammalian tissues. Insulin does not affect adenylate cyclase but enhances the activity of a membrane-bound low $K_m$ cyclic AMP-phosphodiesterase and therefore able to reduce the cyclic AMP content of tissues (109).

Ofulue and Nijjar (110) have indicated that the basal activity of lung particulate adenylate cyclase was depressed in experimental diabetes. However, the enzyme activity of the whole lung homogenate (i.e. including the lung cytosol) was increased, apparently due to increased activity of the adenylate cyclase cytosolic modulator and not to any alteration in the sensitivity of the enzyme to the cytosolic factors. The results indicated about a 2-fold increase in the relative amount of the 65000 dalton component without any change in the amount of the 14500 dalton component (111). Insulin treatment normalized these diabetes-induced changes. In a separate study (112), these investigators observed that experimental diabetes reduced the effective activities of lung calmodulin (as measured by cyclic AMP-phosphodiesterase activation)
and of lung cyclic AMP-phosphodiesterase, both effects apparently due to the presence of a heat-stable calmodulin-inhibitory protein. Interesting was the finding that in the lungs of the diabetic animals there was an apparent shift of calmodulin from the particulate to the cytosolic compartment. This calmodulin shift may be responsible for the reduced basal activity of particulate adenylate cyclase observed in diabetic rat lungs, based on the observation that rat lung particulate fractions contained an adenylate cyclase which was dependent on calmodulin for its activity (58). In the normal rat lung, the activity of adenylate cyclase relative to that of cyclic AMP-phosphodiesterase corresponded approximately with the cyclic AMP levels (23). However this relationship was not maintained in lungs of the experimental diabetic animals (111). The pathophysiologic significance of these findings remained speculative and undefined.

E. Rationale

Studies of the growing lung in diabetes have been lacking until recently. Based on the previous studies that associated alveolar formation with the maturation of the septal network of elastin and collagen, the study of lung growth in experimental diabetes will provide new knowledge since it now appears that altered connective tissue metabolism may be characteristic of diabetes. Experimental diabetes altered the activities of the adenylate cyclase cytosolic modulator and of calmodulin. These endogenous factors may represent the determinant factors of lung growth and connective tissue metabolism in diabetes.
II. SPECIFIC OBJECTIVES OF PRESENT STUDY

The specific objectives were:

1. to determine the effect of experimental diabetes on qualitative and quantitative morphometry of postnatal lung growth and any associated changes in contents of lung protein, RNA, DNA, pulmonary surfactant, collagen and elastin,

2. to determine the effects of experimental diabetes on lung synthesis of DNA, collagen and elastin \textit{in vivo}, and how these related to changes in activities and/or contents of the adenylate cyclase cytosolic modulator, cyclic AMP and calmodulin, and

3. to determine the \textit{in vitro} effects of soluble lung extracts derived from the control and diabetic animals on the synthesis of DNA, collagen and elastin by normal lung tissue, and to further determine the possible involvement of the adenylate cyclase cytosolic modulator and of calmodulin in mediating these changes.
III. EXPERIMENTAL PROCEDURES

A. Chemicals

All reagents used in this study were of analytical grade and purchased, unless stated otherwise, from either Sigma Chemical Company (St. Louis, Missouri, USA) or Fisher Scientific Limited (Fairlawn, New Jersey, USA). [Methyl-³H]thymidine (6.7 Ci/mmole) was purchased from New England Nuclear (Boston, Massachusetts, USA) and U-[¹⁴C]proline (273 mCi/mmole) was from Amersham Corporation (Oakville, Ontario, Canada).

B. Experimental Animals

Several animal models of diabetes mellitus are available for study, including hereditary diabetes in KK mice and streptozotocin-induced diabetes in rats (113). However, the streptozotocin-induced diabetes was chosen in present and previous studies (101,110-112) because it is easier to monitor and control as regards the timing of onset and duration than the hereditary diabetes. Moreover, the drug-induced diabetes does not pose the problem of genetically-associated differences in lung structure and development. Streptozotocin induction of experimental diabetes is specific (113), and the drug has not been documented to have any toxic effect in mammalian lungs.

In this study, a total of 16 timed-pregnant Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Guelph, Ontario, Canada). Delivery of the litters usually occurred within 96 hours of
arrival in the animal housing facilities. Only the male offsprings (usually about 8 from each mother, thus a total of about 128 young rats) were subsequently employed in this study to avoid any sex differences which may confound the interpretation of results. This study chose weaning for the start of the experiments, since suckling rats rendered diabetic by streptozotocin appeared to recover from the disease (114,115) whereas weaned rats remained diabetic throughout postnatal development(110-112,114).

At weaning (day 21 of postnatal life), four rats were randomly chosen from each of the litters and divided equally into two groups such that each group had an equal representation of the littermates. The animals in one group were made diabetic by intraperitoneal injection of streptozotocin (Sigma product # S0130; 7.5 mg/100 g body weight) dissolved under sterile conditions, immediately before injection, in 0.1 M sodium citrate buffer (pH 4.5) at a concentration of 7.5 mg/ml. The animals in the second group were similarly treated with an equal volume of the citrate buffer and served as controls (see Figure 5). All the animals were housed individually in cages designed to prevent coprophagia and the potential for infections. The animals were allowed free access to water and fed standard pellet diet ad libitum. Urine glucose was monitored daily with Diastix (Miles Laboratories, Rexdale, Ontario, Canada). Usually by the seventh day after injection, the streptozotocin-treated rats had urine glucose levels greater than 1.5% and manifested other typical diabetic characteristics such as polyuria,
Figure 5: Flow chart of the procedure used in preparing the experimental animals.

All animals were sacrificed.
polydipsia and reduced body weight. Thereafter, half the animals in the diabetic group received daily subcutaneous injection of 1.0 to 2.5 units of protamine zinc insulin (Connaught Laboratories, Willowdale, Ontario, Canada) depending on daily measurements of urinary output, urine glucose and body weight. This approximate dose generally reduced the polyuria and glycosuria and also resulted in body weight gain in the diabetic animals. At the same time that the insulin treatment was initiated, half the animals in the control group were put on limited diet (approximately 3 to 4 g/100 g body weight per day compared to 12 g/100 g body weight per day determined prior to this time and thereafter) so that their body weights were comparable to those of the untreated diabetic animals. The inclusion of this weight-matched control group in this study was necessary since the nutritional status and/or somatic growth may influence lung growth and thus may likely confound the interpretation of results.

Exactly four weeks after the induction of diabetes (that is, at seven weeks of postnatal age), following an overnight fast, all the animals in the experimental groups were individually anaesthetized by inhalation of ether and the measurements of body weight and nose-tail length were performed. The abdomen of each rat was incised, the aorta was cut and blood samples were taken for the determination of serum glucose. Isotonic saline was then infused through an inferior vena cava catheter. By this method, the lungs became apparently blood-free as the animals bled to death.
C. **Experimental Design**

1. **General:**

   Lungs removed from the experimental animals were used for two types of experiments:
   
a) the quantitation of the *in vivo* changes by morphometric and biochemical analysis; and

b) the preparation of soluble extracts from these lungs for a study of their effects on lung metabolism *in vitro*.

2. **In vivo studies of lung growth**

   Immediately after the sacrifice of the animals as described above, midsternotomy incisions were made, an endotracheal plastic cannula of known weight was inserted and firmly maintained in place with thread. The lungs were lavaged in situ five times through the tracheal catheter with ice-cold isotonic saline, using 5 mL for each lavage. The volume recovered was about 89% of the infused saline solution. The lavage returns were centrifuged at 500 x g for 15 minutes to sediment cells and debris, and the resultant supernatants were stored at -20°C for subsequent extraction. Immediately after lung lavage, the lungs with the attached cannula were removed carefully from the animals and weighed. Since the lungs were too small to allow all the proposed measurements of lung growth in a single animal, the lungs obtained from an individual animal were used either for biochemical or morphometric analysis, or alternatively separated into left lung (for morphometry) and right lung (for biochemistry) by ligature at the right bronchus.
For morphometry, the lungs were degassed and subsequently inflated with 20% phosphate-buffered formalin at a constant transpulmonary pressure of 25 cm H₂O for 76 hours with the lungs submerged in the fixative. After fixation, the trachea was ligated and the fixed lung volume (Vₗ) was determined by water displacement as described by Scherle (116). Essentially, a beaker was partially filled with distilled water and placed on a balance which was adjusted to zero. The fixed lung, wiped off of excess fixative, was then suspended by a thread from the arm of a laboratory stand and completely submerged into the fluid without touching the sides or the bottom of the beaker. Vₗ in cm³ was estimated as equal to the weight in grams indicated on the balance. In circumstances where only the left lung was removed for morphometry, the fresh lung volume of both left and right lungs were first determined intact and then separately prior to the removal of the right lung. By this method, the total fixed lung volume per animal was estimated.

The lungs for biochemical analysis were dissected free of large airways and blood vessels, minced and rinsed several times in PBS. Portions of the lung minces were then homogenized in PBS in a Potter-Elvehjem homogenizer by 25 hand-driven strokes to give 10%(w/v) homogenates which were subsequently divided into 2 mL aliquots and stored at -70°C until analyzed, usually within two weeks. Dry weights of the lung tissues were determined after repeated freeze-drying of portions of the fresh lung minces to constant dry weights.
3. In vivo metabolic studies:

Twenty four hours prior to the termination of the experimental animals, some of these animals were injected intraperitoneally with a single 0.5 mL dose of \([^{3}\text{H}]\)thymidine (150 uCi/100 g body weight). Twelve and twenty four hours to termination, the other experimental animals were similarly injected with \([^{14}\text{C}]\)proline (50 uCi/100 g body weight).

Blood samples were taken from the tail vein of rats injected with \([^{14}\text{C}]\)proline at various time intervals up to 90 minutes and at the time of sacrifice. The clotted blood was centrifuged at 4°C in a clinical centrifuge, the serum was separated and deproteinized by the addition of 3 volumes of ice-cold 6.67% (w/v) TCA. The protein precipitates were removed by centrifugation at 2000 x g for 15 min and the supernatants were subsequently extracted with ether to remove the TCA and finally stored at -20°C for analyses of their specific radioactivities.

The animals were sacrificed as previously described and the lungs were removed, dissected free of large airways and blood vessels, and weighed. Lung homogenates were prepared in ice-cold PBS and stored at -70°C until analyzed for amount and in vivo radioactivities of DNA, collagen and elastin, including total incorporation, and for the lung activities and/or contents of the adenylate cyclase cytosolic modulator, cyclic AMP and calmodulin.
4. In vitro metabolic studies:

Lung homogenates of the experimental animals were prepared as described for the in vivo experiments, and processed as shown in Figure 6. Essentially, the homogenates were centrifuged for 60 min at 15,000 x g, the supernatant fractions were retained and henceforth designated the "soluble lung extracts". Aliquots of the extracts were heated in a 90°C water bath for 5 min, rapidly cooled on ice, then centrifuged at 10,000 x g for 15 min to precipitate the heat-denatured proteins. The supernatants were recovered and designated the "heat-treated soluble lung extracts". Aliquots of the heat-treated soluble lung extracts were lyophilized, and then mixed with three volumes of 50% (w/v) DEAE-cellulose slurry prepared in 20 mM Tris/1 mM imidazole buffer (pH 7.5) containing 1 mM magnesium acetate, 15 mM mercaptoethanol and 0.25 M NaCl. The mixtures were stirred gently for 30 min, centrifuged at 3000 x g for 5 min, and the supernatants were retained and designated the "heat+DEAE-treated soluble lung extracts".

Each of the extracts were dialyzed against two changes of 100 volumes of double distilled water at 4°C for a total of 36 hours using Spectrapor dialysis tubing (Spectrum Medical Industries, Los Angeles, California, USA) with a molecular weight cutoff of 6000-8000 daltons. Protein concentrations in the extracts were determined as described below. The extracts were filter sterilized (0.2 um pore size) and then added to Dulbecco's Modified Eagles Medium containing 100 ug/mL streptomycin sulfate and 100 units/mL penicillin, to give protein
Figure 6: Flow chart of the procedure used in preparing the soluble lung extracts.

Lung Homogenate

15,000 x g, 60 min

Supernatant

SOLUBLE LUNG EXTRACT

90°C, 5 min

10,000 x g, 15 min

supernatant

HEAT-TREATED
SOLUBLE LUNG EXTRACT

DEAE-cellulose, 0.25 M NaCl

3000 x g, 5 min

supernatant

HEAT+DEAE-TREATED
SOLUBLE LUNG EXTRACT
concentrations ranging from zero to 250 µg/mL of medium. These lung extract-modified media were then used in the in vitro incubations of lung minces.

The lung minces used in this series of experiments were obtained from normal 3 week old male Sprague Dawley rats. This age of rats was ideal since this was the same age that diabetes was induced in the experimental animals. Lungs were aseptically removed from the 3 week old rats as described above, dissected free of large airways and blood vessels and chopped into 1 mm³ minces with a McIlwain tissue chopper (Brinkmann Instruments, Westbury, New York, USA). A group of the lung minces (approximately 250 mg wet weight) were submerged in 2.5 mL of the lung extract-modified Dulbecco's medium contained in 25 mL culture bottles and preincubated in a Dubnoff metabolic shaker at 37°C for 30 min in an atmosphere consisting of 95% O₂ and 5% CO₂. The medium was then aspirated and discarded, and replaced with 2.5 mL of identical medium containing 2.5 uCi of [³H]thymidine or 10 uCi of [¹⁴C]proline. The incubation mixtures that had the [¹⁴C]proline added also contained 0.5 mM ascorbic acid, but B-aminopropionitrile was omitted from these incubation mixtures in order to maximize connective tissue crosslinking and thereby minimize degradation. The incubation was continued for 1 to 4 hours. At the end of incubation, the lung minces were washed extensively with ice-cold PBS, blotted on filter paper, and homogenized in PBS as previously described. All homogenates were stored at -70°C until analyzed for total radioactivities and the activities in DNA, collagen and elastin fractions.
D. Analytical Methods

1. **Light microscopic morphometry:**

The fixed lungs were sectioned sagitally and rectangular blocks of tissue, with edges cut at right angles to the surface, were obtained. The tissue blocks were then put in embedding cassettes, immersed in fixative, and photographed. The tissue blocks were subsequently embedded in paraffin and then cut into 5 um thick sections. Three sections were obtained from each block of tissue derived from individual rat lung, and the sections were approximately equidistant from the medial to lateral surface of the lung. The tissue sections were then put on slide and stained with hematoxylin and eosin.

The area of each tissue block (pre-processed tissue) was measured by projecting an enlarged image of the photograph onto a computer-controlled digitizer. The area of the tissue sections (post-processed tissue) was also determined accordingly. By correcting the size with the appropriate magnification factor, the actual area of the pre-processed and post-processed tissue was obtained. The linear shrinkage factors (lsf) for each tissue block during processing were then calculated:

\[
\text{lsf} = \sqrt{\frac{\text{Area of tissue after embedding}}{\text{Area of tissue before embedding}}}
\]
All the slides were coded and the tissue sections were quantitated morphometrically according to Wiebel (117) without any knowledge of the experimental group of animals from which the lungs were derived. Essentially, the morphometric analysis was performed by placing the slide on the mechanical stage of a Wild M501 microscope and the tissue section was viewed on a square grid (108 mm edges) located on the screen of the microscope, using a 10x objective. Within the counting grid were two diagonally-placed crossed test lines, each 138 mm long, and 42 equidistantly distributed test points. Twenty random fields of the tissue sections were determined equidistantly by three imaginary lines running parallel from the top to the bottom of the lung tissue section.

Each test point within the counting grid was classified into one of five categories depending on the histologic component on which it fell, i.e., alveolar air, alveolar duct air, alveolar wall, conducting airways air, and nonparenchyma. Alveoli were considered to be the smallest discrete air spaces encircled by alveolar walls, whereas the cylindrical core of air within alveolar ducts and sacs internal to the mouths of alveoli was referred to as alveolar duct air. Blood vessels, walls of the conducting airways and connective tissue septa were grouped as nonparenchyma. The number of test points for each of the tissue components was expressed as a proportion of the total number of points, and this represented the volume proportion of the tissue structure. There was no correction made for tissue thickness.
The intercepts through alveolar wall structures \( (I_a) \) and those between the mouths of alveoli and alveolar ducts \( (I_d) \) were also counted with the test line. An intersection through an alveolar wall was counted as one intercept, while an intersection that touched but did not pass through an alveolar wall was counted as one-half intercept \( (2) \). Using the point counts and intercepts, calculations were performed as shown in Table II to obtain the morphometric values of mean linear intercept \( (L_m) \), mean chord length of alveoli, mean chord length of alveolar ducts, alveolar surface area, and surface-to-volume ratio. The morphometric data were corrected by the shrinkage factors, and measurements made on the left lung were applied to both lungs.

Since the absolute volume of alveolar air \( (V_a) \) is a product of total number of alveoli \( (N_a) \) and the mean volume of individual alveolus \( (\bar{V}_a) \), and \( \bar{V}_a \) is related to the cubed root of the mean chord length of alveoli \( (I_a) \) by a constant \( (j) \) which depends on the shape of the alveolus \( (\bar{V}_a = jI_a^{3/2}) \), the ratio of \( V_a : I_a^{3/2} \) was estimated to represent \( N_a \) assuming that the shape of the alveoli remained constant in the lungs.
Table II: Calculation of Morphometric Parameters of Lung Growth

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Abbreviations</th>
<th>Calculations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed lung volume</td>
<td>( V_L )</td>
<td></td>
</tr>
<tr>
<td>Volume proportion of</td>
<td>( V_{Va} )</td>
<td>( V_L \times V_{Va} )</td>
</tr>
<tr>
<td>- alveolar air</td>
<td>( V_{Va} )</td>
<td></td>
</tr>
<tr>
<td>- alveolar duct air</td>
<td>( V_{Vd} )</td>
<td>( V_L \times V_{Vd} )</td>
</tr>
<tr>
<td>- alveolar wall</td>
<td>( V_{Ww} )</td>
<td>( V_L \times V_{Ww} )</td>
</tr>
<tr>
<td>- conducting airways air</td>
<td>( V_{Vb} )</td>
<td>( V_L \times V_{Vb} )</td>
</tr>
<tr>
<td>- nonparenchyma</td>
<td>( V_{Vnp} )</td>
<td>( V_L \times V_{Vnp} )</td>
</tr>
<tr>
<td>Total volume of</td>
<td>( V_a )</td>
<td></td>
</tr>
<tr>
<td>- alveolar air</td>
<td>( V_L \times V_{Va} )</td>
<td></td>
</tr>
<tr>
<td>- alveolar duct air</td>
<td>( V_L \times V_{Vd} )</td>
<td></td>
</tr>
<tr>
<td>- alveolar wall</td>
<td>( V_L \times V_{Ww} )</td>
<td></td>
</tr>
<tr>
<td>- conducting airways air</td>
<td>( V_L \times V_{Vb} )</td>
<td></td>
</tr>
<tr>
<td>- nonparenchyma</td>
<td>( V_L \times V_{Vnp} )</td>
<td></td>
</tr>
<tr>
<td>Total length of test line</td>
<td>( L_T )</td>
<td></td>
</tr>
<tr>
<td>Mean linear intercept (um)</td>
<td>( Lm )</td>
<td>( L_T/I_a )</td>
</tr>
<tr>
<td>Mean chord length of alveoli (um)</td>
<td>( I_a )</td>
<td>( L_T \times V_{Va} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( I_a + I_d )</td>
</tr>
<tr>
<td>Mean chord length of alveolar duct (um)</td>
<td>( I_d )</td>
<td>( L_T \times V_{Vd}/I_d )</td>
</tr>
<tr>
<td>Alveolar surface area (cm²)</td>
<td>( S_w )</td>
<td>( 4V_L/Lm )</td>
</tr>
<tr>
<td>( S_w ) per unit lung volume (cm⁻¹)</td>
<td>( S_{Vw} )</td>
<td>( I_a/L_T )</td>
</tr>
</tbody>
</table>

For \( I_a \) and \( I_d \), see text.

\( L_T \) = length of test line (see text).
2. Extraction of protein, RNA and DNA:

The method of extraction used was essentially that described by Wannemacher et al (118) and outlined in Figure 7. Five mL of ice-cold 10% (w/v) TCA was added to 1 mL aliquots of the tissue homogenates and centrifuged at 1000 x g for 5 min. The supernatants were removed and retained for other analyses. The TCA precipitates formed were serially extracted with 5 mL of 95% ethanol saturated with sodium acetate, then with 5 mL of ethanol:ethyl ether (3:1,v/v), and finally with 5 mL of anhydrous ethyl ether. The three solvent systems facilitated the removal of TCA and most of the tissue lipids. The excess ether was removed by drying the precipitates in an air flow hood for 10 min, and the slightly moist precipitates were then incubated in 4 mL of 0.3N KOH solution at 37°C for 60 min. Two mL aliquots of the solubilized samples were removed for protein estimation, while the remainder were acidified with 1 mL of cold 60% (w/v) PCA and centrifuged at 1000 x g for 5 minutes. The resultant supernatants were used for measurement of RNA content, while the PCA precipitates were incubated in 1 mL of 0.5 N PCA at 95°C for 45 minutes and used for estimation of DNA.

3. Estimation of protein content:

Protein was measured by the method of Lowry et al (119) using bovine serum albumin (BSA) dissolved in 0.3 N KOH as standard. The colour reagent (copper tartrate/sodium carbonate solution) was prepared accordingly immediately prior to assay. Fifty uL aliquots of the protein samples in 0.3 N KOH were diluted to 0.4 mL with distilled
Figure 7: Flow chart of the procedure used in extracting protein, RNA and DNA from the lungs.

Lung Homogenate

10% TCA, 4°C

soluble fraction (RESERVED)

precipitate

Ethanol/ether extractions

KOH Digestion

Acidification with PCA, 1000 x g, 5 min

supernatant

precipitate

RNA

95°C in PCA, 45 min; 1000 x g, 5 min

supernatant

DNA
water. Two mL of the color reagent was then added, and the reaction mixtures were allowed to stand at room temperature for 10 min. Then 0.2 mL of 1 N Folin's reagent was added to the reaction mixture and the reaction was allowed to continue for another 30 min again at room temperature. The color which developed was read at 750 nm in a Pye Unicam SP6-550 Spectrophotometer. Protein contents of the samples were then determined from a standard curve prepared from the absorbance readings of the standard BSA processed similarly as the lung samples. The standard curve was linear in the range of 10 to 150 ug protein, and the absorbance readings of the lung samples were all within the linear range.

4. Estimation of RNA content:

RNA was measured as described by Wannemacher et al (118). One mL aliquots of the RNA extracts were diluted to 10 mL with distilled water and the absorbance read at 260 nm in the spectrophotometer. The RNA in the lung samples was estimated from a standard curve prepared from the absorbance of purified calf liver RNA (Sigma type IV) processed similarly as the lung samples. The standard curve was linear in the range of 5 to 35 ug RNA used for sample RNA estimation.

5. Estimation of content and synthesis of DNA:

DNA was measured according to the method of Burton (120) using calf thymus DNA (Sigma) as standard. The standard DNA was processed in identical fashion as the lung samples. One mL aliquots of the DNA
extracts were added to 2 mL of diphenylamine reagent (J.T. Baker Chemical Co., Phillipsburg, New Jersey, USA) and boiled for 10 min. The reaction mixtures were allowed to cool and their absorbances at 600 nm were measured. The DNA content of the lung samples was estimated from a curve prepared from the absorbance of the standard DNA. The standard curve was linear in the range of 50 to 250 ug DNA used for sample DNA estimation.

Specific radioactivity of DNA was estimated by adding 1 mL of the DNA extracts to scintillation medium contained in standard scintillation vials and counting the $[^3H]$ activity. The incorporation of $[^3H]$thymidine into DNA (dpm/mg DNA) was considered to be synonymous with the net rate of DNA synthesis.

6. Extraction of airway and tissue DSPC

Lipids were extracted from the tissue homogenates and from the lung lavages by the method of Bligh and Dyer (121) with 5 volumes of chloroform:methanol (2:1,v/v). Aliquots of standard $[^{14}C]$DSPC were included in each sample for the estimation of recovery. The mixtures were inverted several times and allowed to stand overnight at room temperature. A biphasic system developed in each tube, and the upper aqueous phase was removed as completely as possible with a pipette, leaving the lower chloroform phase which contained the lipids. In the case of the mixtures of tissue homogenate and extracting solvent, filtration through Whatman No. 1 filter paper on Coors No. 3 Buchner funnel with slight suction was performed prior to separation of the
biphasic system. All extracts were dried under \(N_2\), dissolved in chloroform:methanol (2:1,v/v) and washed with 0.2 volumes of 0.05 M NaCl. The final lipid extracts were then diluted to 5 mL with chloroform:methanol (2:1,v/v). Aliquots of these extracts were transferred to standard pyrex culture tubes with teflon-lined screw caps and DSPC was isolated from these samples by the method described by Mason et al (122). Essentially, the lipid extracts were dried under \(N_2\), the residues were re-dissolved in 0.5 mL of osmium tetroxide-carbon tetrachloride solution (0.1 g/mL) and allowed to stand for 15 min at room temperature. Evaporation of the reactants was then performed in a fume hood and the residues were applied to aluminium oxide columns formed by placing 0.8 g of \(110^\circ\text{C}\)-activated aluminium oxide (100-200 mesh, BioRad Laboratories, Richmond, California, USA) on glass wool plugs in the neck of disposable 9 inch pasteur pipettes. The first elutions with 10 mL of chloroform:methanol (20:1,v/v) contained the neutral lipids and were discarded. DSPC was eluted then with 5 mL of chloroform:methanol:7 M ammonium hydroxide (70:30:2, v/v/v) and collected in hydrolysis tubes. The DSPC extracts were all reduced to approximately 2 mL under \(N_2\).

7. Estimation of DSPC content:

The total phosphorus in the DSPC extracts was estimated by a modified method of Bartlett (123). Essentially, 0.5 mL of 10 N \(\text{H}_2\text{SO}_4\) was added to each hydrolysis tube containing 1 mL of the DSPC extracts and the mixtures were digested in a 160\(^\circ\text{C}\) oven for 5 hours. After cooling, 4.4 mL of water plus 0.2 mL of 5% (w/v) ammonium molybdate and
0.2 mL of the reducing agent (containing 15 g of sodium bisulfite, 0.5 g of sodium sulfite, and 0.25 g of l-amino-2-naphthol-4-sulfonic acid) were added, mixed thoroughly, and heated for 20 min with the tubes capped. The absorbance at 660 nm was then measured with the spectrophotometer. Estimation of the phosphorus content in the lung DSPC extracts was accomplished by comparing the absorbance readings to a standard curve prepared from the absorbance of phosphorus standards (Pierce Chemical Company, Rockford, Illinois, USA). The standard curve was linear in the range of 0.5 to 5.0 ug phosphorus that was used for sample estimation. The quantity of DSPC in lung samples was assumed to be 25 times the phosphorus content.

8. Extraction of collagen and elastin:

The extraction procedure employed was identical to the method described by Laurent et al (124) in which cyanogen bromide essentially solubilized all the lung collagen leaving an insoluble pellet with an amino acid content similar to elastin. This procedure is depicted in Figure 8. In the present study, 2 mL of lung homogenates contained in 10 mL glass Potter-Elvehjem homogenizers were centrifuged at 4000 x g for 5 min and the supernatants retained. The residues were washed twice in 2 mL of PBS, resuspended in 5 mL of 2% (w/v) sodium dodecyl sulfate (SDS), re-homogenized and centrifuged. This procedure was repeated four times. All supernatants were pooled, the proteins were precipitated in 5% TCA and retained for hydroxyproline and protein estimation. The remaining residues were washed three times in 5 mL of PBS and then twice in 2 mL of
Figure 8: Flow chart of the procedure used in extracting collagen and elastin from the lungs.

1. **Lung Homogenate**
   - 4000 x g, 5 min
     - supernatant
     - precipitate
       - 2% SDS
       - residues
         - CNBr digestion, 37°C, 10 hrs
           - 5000 x g, 15 min
             - supernatant
             - precipitate
               - COLLAGEN
               - ELASTIN
acetone. The washed residues were dried under vacuum, re-homogenized in 0.75 mL of 70% (w/v) formic acid, and then transferred to graduated polythene tubes. The homogenizers were washed with another 0.75 mL of the formic acid and the samples added to those in the polythene tubes. The final volumes were made to 2 mL with the formic acid, and 0.5 mL of a 0.1 g/mL solution of cyanogen bromide (CNBr) was added to produce a final CNBr concentration of 20 mg/mL. Nitrogen gas was bubbled through the samples, the tubes were sealed and positioned at inclined angles in a test tube rack placed in a shaking water bath set at 37°C. The reaction was allowed to proceed for 5 hours, and the resulting digests were centrifuged at 5000 x g for 15 min. The supernatants were pipetted out and retained. Another CNBr digestion was repeated, and the supernatants were pooled, diluted 10-fold with distilled water and lyophilized. These CNBr-solubilized materials represented the collagen fractions. Finally, the residues after CNBr digestion (representing the elastin fractions) were washed twice in distilled water and lyophilized to constant weight.

9. **Estimation of hydroxyproline content:**

Samples recovered from the SDS/PBS extraction and CNBr digestion (namely soluble protein, collagen and elastin, respectively) were hydrolyzed in 6 N HCl (approximately 1 mL for each 25 mg dry weight) to liberate hydroxyproline from peptide linkage. Hydrolysis was performed in hydrolysis tubes with Teflon-lined screw caps at 110°C for 18 hours. After hydrolysis, the hydrolyzates were evaporated on a
heating module (Reacti-Therm Evaporating Unit, model 18780, Pierce Chemical Co., Rockford, Illinois, USA) with a jet of air directed into the tubes to speed evaporation of the acid. The dried hydrolyzates were dissolved in 5 mL of distilled H₂O and dried again. The evaporation was repeated 3-4 times and the samples were then made up to known volumes with distilled H₂O.

Hydroxyproline was measured in the hydrolyzates using the spectrophotometric method described by Woessner (125). Essentially, 2 mL aliquots of the hydrolyzates and also a series of standards containing 0-5 ug L-hydroxyproline in 2 mL total volume of 0.001 N HCl were placed in standard assay tubes. One mL of freshly prepared chloramine-T solution was added to each tube and allowed to stand for 20 min at room temperature. Then 1 mL of 0.7% (w/v) PCA was added, mixed and left to stand for another 5 min. Finally, 1 mL of Ehrlich's reagent (20 g of p-dimethylaminobenzaldehyde in 100 ml of ethylene glycol monomethyl ether) was added, mixed vigorously, and heated for 20 min in a 60°C water bath. The tubes were cooled in tap water for 5 min and the absorbance of the samples was read in the spectrophotometer at 561 nm. The standard curve prepared was linear to 5 ug hydroxyproline, and the absorbance of the lung samples were all within the linear range. The hydroxyproline contents of the lung samples were estimated directly from the standard curve.
10. Determination of proline and hydroxyproline activities:

The separation of proline and hydroxyproline was achieved by Dowex 50-X12 (200-400 mesh, BioRad Laboratories, Richmond, California, USA) chromatography in columns (1.0 x 2.4 cm) equilibrated at room temperature with 1 N HCl. Standard L-[¹⁴C]proline (0.40 uCi) and L-[³H]hydroxyproline (0.48 uCi) in 0.2 mL of 0.001 N HCl were first applied to the columns and eluted with 1N HCl at a flow rate of 25 mL/hour, and 5 mL fractions were collected. The eluted fractions were dried to remove the HCl, re-suspended in 1.5 mL of distilled water and mixed with 10 mL of scintillation medium. Radioactivity was determined by double-channel counting in the liquid scintillation counter. Based on the radioactivity measured, the elution positions of proline and hydroxyproline were established for each batch of resin. Thereafter, aliquots of deproteinized serum, TCA-soluble extracts of lung homogenates, or hydrolyzates derived from the soluble protein, collagen and elastin fractions of lung, were placed on the columns and similarly eluted. The fractions representing proline and hydroxyproline were pooled accordingly, dried by evaporation and re-suspended in known volumes of distilled water. Radioactivity was determined in 1 mL aliquots by liquid scintillation counting. Aliquots of each sample were also analyzed for proline content on a Technicon amino acid analyzer (Biochemistry Dept.) and for hydroxyproline content by spectrophotometry. Specific radioactivities were estimated as dpm/umole of proline and as dpm/ug of hydroxyproline.
11. Estimation of content and synthesis of collagen and elastin:

Collagen contents were calculated from hydroxyproline estimates of the CNBr-solubilized lung samples. Laurent et al (124) have indicated that lung collagen contains 12.2% (w/w) hydroxyproline based on amino acid analysis of collagen standards and lung tissue collagen extracted in CNBr. Thus, the quantity of collagen was determined accordingly by using the formula:

\[ \text{ug collagen} = 8.2 \times \text{ug hydroxyproline} \]

Elastin contents were estimated in the CNBr-insoluble fractions of lung samples by gravimetric measurements after lyophilization to constant weight and also according to Naum and Morgan (126). Essentially, 1 mL of highly purified elastase solution (0.1 mg/mL Sigma Type III elastase, from porcine pancreas, in 0.02 M NaHCO_3 buffer pH 8.8) was added to the samples, and the mixtures were incubated for 30 min at 37°C in a shaking water bath. Aliquots of the elastase digests were removed for protein assay by the method of Lowry et al (119), and elastin contents were quantitated as equal to protein contents. Suspensions of purified collagen and elastin standards, bovine serum albumin, rat blood and rat lung cytosol were also assayed simultaneously in order to determine specificity of the elastin assay method. Both the gravimetric and elastase-derived estimates of lung elastin approximated each other.

The in vivo biosynthesis of collagen and elastin were estimated by the incorporation of radiolabeled proline into the lung connective
tissue proteins. The synthesis of elastin was taken to equal dpm of the CNBr-insoluble materials/mg DNA and thus represented total radioactivity of proline and its metabolites. However, the synthesis of collagen was taken to equal dpm of hydroxyproline in the CNBr-solubilized materials/mg DNA. The specific radioactivities of proline in the lung tissue-free pool and in serum were used to correct for the availability of the labelled precursor in the lung tissue. For this purpose, the ratio of the areas under the curves of specific activity of serum proline with times after injections of normal control and the other experimental animals were calculated, and the incorporation values obtained were multiplied by this factor.

In vitro synthesis of elastin was also calculated as dpm of elastin fractions/mg DNA and that of collagen as dpm of hydroxyproline in collagen fractions/mg DNA. However, values were only corrected with the specific activity of proline in the tissue-free pool.

12. Estimation of cyclic AMP content:

Five hundred uL aliquots of the supernatants recovered from lung homogenates after TCA precipitation (see Extraction of protein, RNA and DNA) were mixed with 5 mL of ethyl ether saturated with H₂O, and a biphasic system was allowed to form. The upper ether phase was removed and discarded, and the lower aqueous phase was extracted two additional times with ethyl ether and then evaporated to dryness under a stream of air in a 65°C water bath. The residues were subsequently dissolved in
1 mL of 0.05 M sodium acetate buffer (pH 6.2), and 25, 50, and 100 µL aliquots were transferred into disposable polypropylene tubes and then assayed for cyclic AMP using the Becton-Dickinson (Orangeburgh, New York, USA) cyclic AMP radioimmunoassay kit. This radioimmunoassay kit contains an antibody which is highly specific for cyclic AMP and thus permits the direct measurement of cyclic AMP without preliminary separation of the other nucleotides (127). In this assay, the tracer $^{125}$I cyclic AMP is displaced from the antibody by increasing amounts of cyclic AMP in the standard or samples during incubation at 4°C for 20 hours. Separation of bound cyclic AMP was achieved rapidly by the addition of 60% (w/v) saturated ammonium sulfate. A standard curve was plotted with counts derived from the cyclic AMP standard, and from it the contents of cyclic AMP in the lung samples were determined.

13. **Estimation of adenylate cyclase cytosolic modulator activity:**

The activity of the adenylate cyclase cytosolic modulator was measured by the ability of the lung cytosolic fraction to enhance the basal adenylate cyclase activity present in a standard membrane preparation of lung tissue, essentially as described by Ofulue and Nijjar(58,110). Standard lung membrane fractions were prepared from lung homogenates in 10 mM Tris-HCl/0.27 M sucrose buffer (pH 7.4) by centrifugations at 1000 x g for 10 min (to remove unbroken cells and debris) and at 100,000 x g for 60 min. The supernatants were withdrawn and the pellets were washed twice by re-suspension in five volumes of the homogenization buffer. The final pellets were re-suspended in 10 mM
Tris-HCl (about 2 mg protein/mL). The enzyme activity was then measured by the method of Drummond and Duncan (128) using [8-\textsuperscript{14}C]ATP as substrate. The reaction medium (90\textmu L) contained 40 mM Tris-HCl buffer (pH 7.5), 25 mM caffeine, 5.5 mM KCl, 10 mM MgCl\textsubscript{2}, 20 mM phosphoenolpyruvate, 130 ug/mL of pyruvate kinase, 2 mM cyclic AMP, 50-75 ug protein of the standard lung membrane preparation and varying amounts of soluble lung extracts. The reactants were preincubated at 37\textdegree C for 3 min, and the reaction was started by the addition of 10 \textmu L of \textsuperscript{14}C]ATP (10 uCi, 0.56mM). Some reaction mixtures were boiled prior to the addition of \textsuperscript{14}C]ATP for estimation of background counts. The reaction was allowed to run for 10 min, and then stopped by immersing the reaction tubes in boiling water for 3 min. The mixtures were centrifuged at 1000 x g for 15 min at 4\textdegree C and 75 \textmu L of the clear supernatants were then applied to Whatmann No. 3 MM filter papers which had cyclic AMP standard applied for identification of cyclic AMP spots under ultraviolet light. Descending chromatography was performed for 18 hours at room temperature in a solvent containing 1 M ammonium acetate-95% ethanol (3:7, v/v). The papers were air dried, the cyclic AMP spots were cut out and placed in 18 mL of scintillation fluid. The radioactivity was counted and corrections were made for background radioactivity. The amount of cyclic AMP formed was calculated from the specific activity of the \textsuperscript{14}C]ATP used as substrate and represented adenylate cyclase activity. The differences in enzyme activity in the absence and presence of the cytosolic lung extracts represented the activity of the adenylate cyclase cytosolic modulator.
14. Estimation of immunoreactive calmodulin content:

Calmodulin was measured with a radioimmunoassay kit (Amersham Corporation, Oakville, Ontario, Canada) which contains an affinity purified antibody that has a high specificity and affinity for calmodulin. The assay is based on the competition between unlabelled calmodulin and a fixed quantity of \(^{125}\text{I}\)calmodulin to the antibody, hence the amount of antibody-bound \(^{125}\text{I}\)calmodulin is inversely related to the amount of calmodulin present in the assay sample. In this study, prior to calmodulin estimation, 0.5 mL aliquots of lung homogenates were lyophilized and the dried samples were subsequently redissolved in 0.5 mL of 125 mM sodium borate buffer (pH 8.4) containing 1 mM EGTA and 75 mM sodium chloride and then heated at 90°C for 5 min. The heat-treated samples were rapidly cooled at 4°C and centrifuged at 10,000 x g for 30 min and the supernatants were recovered for calmodulin estimation. The calmodulin standard (5 ug, provided in the RIA kit) was dissolved in 2 mL of the sodium borate buffer and similarly treated, and the resultant supernatant was serially diluted as recommended in the RIA kit. The lung samples were diluted 10- to 20-fold with the assay buffer and 0.2 mL aliquots were assayed for calmodulin. All assays were performed in duplicate in polystyrene tubes for 20 hours at room temperature. Separation of the antibody-bound calmodulin from unbound calmodulin was achieved by precipitation with formalin-fixed Staphylococcus aureus (Staph A reagent) followed by centrifugation. The pellets were washed with buffer and counted. A standard curve was prepared from counts derived with the calmodulin standard, and from it the amounts of calmodulin in the lung samples were determined.
15. Estimation of calmodulin activity:

The method of calmodulin assay was essentially as described by Sharma and Wang (129). The calmodulin activities of different amounts of appropriately diluted heat-treated lung samples were assayed by measuring their stimulation of calmodulin-deficient phosphodiesterase in the absence and presence of 0.17 mM calcium with or without 0.17 mM EGTA. In a total volume of 0.9 mL, the assay medium also contained 40 mM Tris/40 mM imidazole buffer (pH 7.5) and 3 mM magnesium acetate, 0.1 mL of 5'-nucleotidase (0.25 U, Sigma grade III) dissolved in 10 mM Tris-HCl containing 0.5 mM magnesium acetate (pH 7.5), 0.012 units of the standard phosphodiesterase (Sigma product # P9529), and varying amounts of the lung samples. The reaction was initiated, after pre-incubation for 3 min at 30°C, by the addition of cyclic AMP (1.2 mM final concentration), allowed to continue for 30 min and then stopped by adding 0.2 mL of ice-cold 55% (w/v) TCA. Assay control mixtures were similarly treated except that the TCA was added before the cyclic AMP to inhibit any reaction. The reaction mixtures were finally centrifuged to sediment the denatured proteins. The liberated inorganic phosphate was then quantitated by adding 500 uL aliquots of the clear supernatant to 0.5 mL of 55% ammonium molybdate in 1.1 N H$_2$SO$_4$, followed by the addition of 0.05 mL of reducing agent containing 12 g of sodium bisulfite, 1.2 g sodium sulfite and 0.25 g of 1-amino, 2-naphtol, 4-sulfonic acid in 100 mL of deionized water. The color reactions were allowed to stabilize for 7 minutes at room temperature. The absorbance was read at 660 nm and used as estimate of calmodulin activity.
16. Sephadex G-75 column chromatography:

Ten grams of Sephadex G-75 powder (Pharmacia Fine Chemicals, Piscataway, New Jersey, USA) was soaked in 2 L of distilled water, allowed to swell at 90°C for 3 hours, and then washed in a Buchner funnel with more than 10 L of hot distilled water until the pH was close to neutrality. The gel was then resuspended in PBS and packed into 500-mL bed volume columns (2 x 84 cm) equilibrated in PBS at 4°C. Aliquots of soluble lung extracts or samples derived from them were applied onto the columns and eluted with PBS at a flow rate of 6 mL/hour. Five mL fractions were collected and monitored for protein by spectrophotometry at 280 nm. The columns were also calibrated with proteins of known molecular weights (ovalbumin, 45000 daltons; chymotrypsinogen A, 25000 daltons; calmodulin, 17000 daltons; ribonuclease A, 13700 daltons), and the void volumes were determined with blue dextran (2 x 10^6 daltons). The eluted fractions of the lung samples were lyophilized and resuspended in Dulbecco's medium for the in vitro metabolic studies.

17. Fluphenazine-Sepharose affinity chromatography:

Fluphenazine was coupled to Sepharose according to the procedure described by Kakiuchi et al (130) under reduced lighting conditions. Epoxy-activated Sepharose 6B (Sigma) was soaked with double-distilled water and washed, on a sintered glass funnel, with reaction medium consisting of 0.1 M sodium carbonate buffer (pH 11.0) and dioxane at 20% (v/v) final concentration. Ten mL of the washed Sepharose
was then mixed with 800 mg of Fluphenazine (Squibb and Sons Inc., Princeton, New Jersey, USA) in 40 mL of the reaction medium, and the pH was maintained at 11.0 by the addition of NaOH. The reaction mixture was then incubated in a shaking water bath for 48 hours at 50°C. At the end of incubation, the resultant Fluphenazine-coupled Sepharose slurry was washed with the reaction medium, with double-distilled water, and then in 5 resin volumes of 1 M ethanolamine (pH 8.0) with subsequent filtrations on the sintered glass funnel. The ethanolamine treatment was repeated several times for a total of 3.5 hours, and the Fluphenazine-Sepharose was washed with double-distilled water and finally with 10 mM Tris-HCl (pH 7.5).

Aliquots of samples derived from the soluble lung extracts were then applied to columns (1 x 2 cm) of the Fluphenazine-Sepharose equilibrated at 4°C with the Tris-HCl buffer that contained 50 mM NaCl and 0.2 mM CaCl₂. The columns were then eluted with the equilibration buffer, 1 mL fractions were collected and their absorbances at 280 nm were subsequently measured. When the absorbance readings of the column eluents were relatively zero, the elution medium was switched to that containing, in addition, 2 mM EGTA. Further 1 mL fractions were collected and monitored also for protein by spectrophotometry at 280 nm until the elution of bound protein materials from the columns was completed. All the eluted fractions were dialyzed extensively against double-distilled water, lyophilized, and then resuspended in PBS at half the original volume. Aliquots of these fractions were then added to incubation medium for the lung in vitro metabolic studies.
E. **Determination of Radioactivity**

Radioactivity of $[^3H]$- and $[^{14}C]$-labelled samples were measured with a Philips liquid scintillation counter (Model PW 4700; Philips Company, Holland). The scintillation medium utilized was the universal liquid scintillation counting cocktail, ScintiVerse (Fisher), containing all the necessary fluors and solvents. The aliquots for assay of radioactivity were chosen so that the measured counts were at least ten times the background. All counts were automatically converted to disintegration per minute (dpm) using internal efficiency corrections for quenching. Radioactivities of $[^{125}I]$cyclic AMP and $[^{125}I]$calmodulin were measured with an LKB universal gamma counter (1282 Compugamma).

F. **Statistical Analysis of Data**

In the assessment of all measured values, a single factor analysis of variance (ANOVA) was used to test the hypothesis that the means of the different experimental groups were equal. If the hypothesis was rejected at the 1% significance level, then the Student-Newman-Keuls (SNK) multiple range test was performed at the 5% significance level to determine significant differences between the experimental groups (131).
A. General Characteristics

The general characteristics of the experimental animals are shown in Table III. The streptozotocin-treated rats were markedly hyperglycemic and manifested typical characteristics of insulin-deficiency diabetes mellitus such as polydipsia, polyuria and glycosuria, and thus confirmed the establishment of experimental diabetes in these animals. Insulin treatment of the diabetic rats reversed these diabetic characteristics towards control values. In addition, the experimental diabetic rats were polyphagic and gained less weight, about 75% of the normal control body weights. Since the body size of an animal, in part determined by body weight, influences lung growth (1,6,75,77), it was important to include in this study a group of control rats undernourished and thus weight-matched for the untreated diabetic rats. Nose-tail length, another index of body size, was also reduced in the untreated diabetic rats but was not different from the undernourished control values. Insulin-treated diabetic and normal control animals were not different in the measured body size parameters.
### Table III: General Characteristics of the Experimental Animals

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Normal Control</th>
<th>Undernourished Control</th>
<th>Untreated Diabetic</th>
<th>Insulin-treated Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>BW (g)</td>
<td>229 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>169 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>172 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>217 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NTL (cm)</td>
<td>31.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.9 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.3 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O consumption (mL/day)</td>
<td>69 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>302 ± 15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>173 ± 8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>mL urine/day</td>
<td>17 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>135 ± 9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>51 ± 4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum glucose (mg/dL)</td>
<td>106.6 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>102.0 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>369.7 ± 2.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>197.2 ± 2.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urine glucose (%)</td>
<td>Negative&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Negative&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 - 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1 - 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

BW = Body weight; NTL = Nose-tail length

Values are mean ± SEM;

<sup>a,b,c,d</sup> In each horizontal row, means without a common superscript are significantly different (P<0.05) by analysis of variance and SNK multiple range test.
B. Changes in Lung Weight and Lung Volume

As shown in Table IV, lung weight was reduced by 25% in the undernourished controls but only by 15% in the untreated diabetic animals. Thus the lung weight:body weight ratio (the specific lung weight) was similar in the control animals but increased in the untreated diabetic rats, and suggest that lung tissue growth was maintained and possibly increased in experimental diabetes, regardless of impaired body growth. The changes in lung weight did not appear to be due to water content since there were no significant differences in wet:dry weight ratios of the lungs in the experimental animals. The volume of lungs fixed at 25 cm H₂O pressure was 25% and 20% lower in the undernourished control and untreated diabetic animals, respectively. Hence, the lung volume per unit body weight (the specific lung volumes) was the same in the control animals and slightly but significantly increased in the untreated diabetic rats. The fixed lung volume:lung weight ratios in the untreated diabetic animals were 85% and 81% of normal control and undernourished control values, respectively. The volume of fixative per unit lung weight in the untreated diabetic animals was 90% of the normal control values and 85% of the values in the undernourished control animals. Insulin treatment of the diabetic animals normalized the changes in absolute and specific values of lung weight and lung volume, but the lung volume:lung weight ratios and the volume of fixative per unit of lung weight were midway between the values of the untreated diabetic and normal control animals.
Table IV: Experimental Diabetes-induced Changes in Lung Weight and Lung Volume.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Normal Control</th>
<th>Undernourished Control</th>
<th>Untreated Diabetic</th>
<th>Insulin-treated Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>W_L (g)</td>
<td>1.08 ± 0.05\textsuperscript{a}</td>
<td>0.78 ± 0.07\textsuperscript{b}</td>
<td>0.91 ± 0.06\textsuperscript{c}</td>
<td>1.12 ± 0.09\textsuperscript{a}</td>
</tr>
<tr>
<td>W_L/dry Lung wt (g/g)</td>
<td>4.85 ± 0.06</td>
<td>4.79 ± 0.10</td>
<td>4.90 ± 0.11</td>
<td>4.89 ± 0.10</td>
</tr>
<tr>
<td>W_L/100g BW</td>
<td>0.477 ± 0.005\textsuperscript{a}</td>
<td>0.472 ± 0.008\textsuperscript{a}</td>
<td>0.530 ± 0.009\textsuperscript{b}</td>
<td>0.482 ± 0.007\textsuperscript{a}</td>
</tr>
<tr>
<td>V_L (cm\textsuperscript{3})</td>
<td>9.30 ± 0.09\textsuperscript{a}</td>
<td>6.90 ± 0.05\textsuperscript{b}</td>
<td>7.15 ± 0.11\textsuperscript{c}</td>
<td>9.10 ± 0.12\textsuperscript{a}</td>
</tr>
<tr>
<td>V_L/100g BW</td>
<td>3.99 ± 0.07\textsuperscript{a}</td>
<td>4.03 ± 0.09\textsuperscript{a}</td>
<td>4.34 ± 0.08\textsuperscript{b}</td>
<td>4.05 ± 0.09\textsuperscript{a}</td>
</tr>
<tr>
<td>V_L/W_L (cm\textsuperscript{3}/g)</td>
<td>8.51 ± 0.07\textsuperscript{a}</td>
<td>8.86 ± 0.09\textsuperscript{b}</td>
<td>7.25 ± 0.10\textsuperscript{c}</td>
<td>8.19 ± 0.10\textsuperscript{d}</td>
</tr>
<tr>
<td>Fixative/W_L</td>
<td>7.67 ± 0.05\textsuperscript{a}</td>
<td>7.90 ± 0.08\textsuperscript{b}</td>
<td>6.91 ± 0.12\textsuperscript{c}</td>
<td>7.20 ± 0.11\textsuperscript{d}</td>
</tr>
</tbody>
</table>

W_L = Wet Lung weight; V_L = Fixed Lung volume; Fixative/W_L = \( V_L - \frac{W_L}{1.06} \)

where 1.06 is equal to the density of the lung.

Values are mean ± SEM

\textsuperscript{a,b,c,d}In each horizontal row, means without a common superscript are significantly different (p<0.05) by analysis of variance and SNK multiple range test.
C. Changes in DNA, Protein, RNA and Connective Tissue proteins

As shown in Table V, the DNA content per gram of lung tissue in the untreated diabetic animals was 92% of the normal control values but was 84% of the values in the undernourished control animals. However, the total DNA content was similar to those of the undernourished controls but was 75% of the normal control values. The lung content of DNA per unit of body weight was not significantly altered in the undernourished control and untreated diabetic animals. Insulin treatment of the diabetic animals completely reversed the changes in absolute and specific DNA content of the lungs to normal control values but caused an increase in the DNA content per gram of lung tissue.

The protein:DNA ratio in the lungs of the untreated diabetic animals was similar to the values in the normal control lungs but was 131% of the values in the undernourished control animals. Similarly, the proportion of non-connective tissue proteins to DNA in the lungs of the untreated diabetic and the normal control animals were not significantly different but were greater than the values of the undernourished control animals. The RNA:DNA ratio was similarly altered. The insulin-treated diabetic animals had lung protein:DNA and RNA:DNA ratios higher than those of the normal control animals.

The collagen content per gram of lung tissue in the untreated diabetic animals was 130% of the normal control values and 154% of the values in the undernourished control animals. The total collagen content
per lung was 110% and 180% of the normal control and undernourished control values, respectively. The total content of lung collagen per unit of body weight was 147% of the normal control values and 178% of the values in the undernourished control animals. The collagen:DNA ratio in the lungs of these diabetic animals was 139% of the values in the normal control animals and 183% of the undernourished control values. All the values of collagen content in the insulin-treated diabetic animals were midway between those of the untreated diabetic animals and the normal control group, except for the absolute amount which was further increased.

The elastin content per gram of lung tissue in the untreated diabetic group was 127% and 151% of the normal control and undernourished control values, respectively. The total elastin content per lung was 107% of the normal control values and 170% of the values in the undernourished control group. The total content of lung elastin relative to body weight was 145% of values in the normal control group and 173% of the undernourished control values. The elastin:DNA ratio in the lungs of these diabetic animals was 138% and 171% of the normal control and undernourished control animals, respectively. Insulin treatment of the diabetic animals partially restored the elastin content per gram of lung tissue and the total elastin content relative to body weight, did not affect the absolute amount, and completely normalized the elastin:DNA ratio.
Table V: Experimental Diabetes-induced Changes in Biochemical Aspects of Lung Growth

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Normal Control</th>
<th>Undernourished Control</th>
<th>Untreated Diabetes</th>
<th>Insulin-treated Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=15</td>
<td>n=15</td>
<td>n=15</td>
<td>n=15</td>
</tr>
<tr>
<td>DNA: mg/g tissue</td>
<td>4.47 ± 0.10</td>
<td>4.69 ± 0.15</td>
<td>4.12 ± 0.11</td>
<td>4.60 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>mg/Lung</td>
<td>4.83 ± 0.10</td>
<td>3.82 ± 0.13</td>
<td>3.75 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>mg/Lung/100 g BW</td>
<td>2.14 ± 0.08</td>
<td>2.20 ± 0.13</td>
<td>2.18 ± 0.10</td>
</tr>
<tr>
<td>PROTEIN/DNA: mg/mg</td>
<td>26.42 ± 1.32</td>
<td>20.75 ± 1.09</td>
<td>27.19 ± 1.21</td>
<td>29.18 ± 1.17</td>
</tr>
<tr>
<td>NCT-PROTEIN*/DNA:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/mg</td>
<td>22.35 ± 1.12</td>
<td>17.60 ± 0.99</td>
<td>21.43 ± 1.25</td>
<td>24.88 ± 1.14</td>
</tr>
<tr>
<td>RNA/DNA: mg/mg</td>
<td>0.51 ± 0.03</td>
<td>0.40 ± 0.02</td>
<td>0.55 ± 0.05</td>
<td>0.59 ± 0.03</td>
</tr>
<tr>
<td>COLLAGEN: mg/g tissue</td>
<td>15.21 ± 0.91</td>
<td>12.82 ± 0.80</td>
<td>19.73 ± 1.06</td>
<td>16.53 ± 0.73</td>
</tr>
<tr>
<td></td>
<td>mg/Lung</td>
<td>16.35 ± 0.10</td>
<td>9.95 ± 0.08</td>
<td>18.02 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>mg/Lung/100 g BW</td>
<td>7.15 ± 0.20</td>
<td>5.90 ± 0.13</td>
<td>10.54 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>mg/mg DNA</td>
<td>3.42 ± 0.09</td>
<td>2.59 ± 0.06</td>
<td>4.75 ± 0.11</td>
</tr>
<tr>
<td>ELASTIN: mg/g tissue</td>
<td>3.10 ± 0.08</td>
<td>2.62 ± 0.06</td>
<td>3.95 ± 0.11</td>
<td>3.25 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>mg/Lung</td>
<td>3.34 ± 0.05</td>
<td>2.10 ± 0.07</td>
<td>3.58 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>mg/Lung/100 g BW</td>
<td>1.44 ± 0.05</td>
<td>1.21 ± 0.03</td>
<td>2.09 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>mg/mg DNA</td>
<td>0.68 ± 0.04</td>
<td>0.55 ± 0.03</td>
<td>0.94 ± 0.06</td>
</tr>
</tbody>
</table>

*NCT-Protein = Non-connective tissue proteins.

Values are mean ± SEM.

a,b,c,d In each horizontal row, means without a common superscript are significantly different (P<0.05) by analysis of variance and SNK multiple range test.
D. Changes in Quantitative and Qualitative Morphometry

As shown in Table VI, quantitative morphometric analysis by point counts demonstrated a reduction in the volume proportion of alveolar air with a concomitant increase in the proportion of alveolar wall in the lungs of the untreated diabetic animals. Insulin treatment of the diabetic animals failed to completely reverse these changes. There were no significant differences between the control and diabetic animals with regards to the volume proportions of alveolar duct air, conducting airway air and nonparenchymal tissues. The undernourished control animals had similar values as the normal control group.

As shown in Table VII, the absolute volumes of alveolar air and of alveolar duct air in the lungs of the untreated diabetic animals were about 84% of normal control values but were not different from those of the undernourished controls. The absolute volume of alveolar wall in the lungs of the diabetic rats was 10% and 35% higher than the normal control and undernourished control values, respectively. Insulin treatment of the diabetic animals normalized the change in alveolar duct, partially reversed that of the alveolar air but increased further that of the alveolar wall. Specific volumes of alveolar air and alveolar duct air in the undernourished control and untreated diabetic animals were similar to normal control values. However, that of alveolar wall in the untreated diabetic animals was 147% of the control values. Insulin therapy partially reversed this change.
Table VI: Effect of Experimental Diabetes on Volume Proportions of Lung Structures.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Normal Control</th>
<th>Undernourished Control</th>
<th>Untreated Diabetic</th>
<th>Insulin-treated Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>V_{VA}</td>
<td></td>
<td>0.520 ± 0.005^a</td>
<td>0.516 ± 0.009^a</td>
<td>0.477 ± 0.006^b</td>
</tr>
<tr>
<td>V_{VD}</td>
<td></td>
<td>0.251 ± 0.011</td>
<td>0.264 ± 0.023</td>
<td>0.259 ± 0.019</td>
</tr>
<tr>
<td>V_{VW}</td>
<td></td>
<td>0.143 ± 0.009^a</td>
<td>0.148 ± 0.011^a</td>
<td>0.199 ± 0.013^b</td>
</tr>
<tr>
<td>V_{VB}</td>
<td></td>
<td>0.026 ± 0.006</td>
<td>0.028 ± 0.010</td>
<td>0.029 ± 0.011</td>
</tr>
<tr>
<td>V_{VNP}</td>
<td></td>
<td>0.060 ± 0.008</td>
<td>0.065 ± 0.007</td>
<td>0.064 ± 0.012</td>
</tr>
</tbody>
</table>

*Volume proportion of alveolar air = V_{VA}; of alveolar duct air = V_{VD}; of alveolar wall = V_{VW}; of conducting airways = V_{VB}; of nonparenchyma = V_{VNP}.*

Values are mean ± SEM.

^a,b,c^ Means in each horizontal row without a common superscript are significantly different (P<0.05) by analysis of variance and SNK multiple range test.
Table VII: Effect of Experimental Diabetes on Absolute and Specific Volumes of Lung Parenchymal Structures.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Normal Control</th>
<th>Undernourished Control</th>
<th>Untreated Diabetic</th>
<th>Insulin-treated Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>$V_a$</td>
<td>$4.83 \pm 0.09^a$</td>
<td>$3.49 \pm 0.06^b$</td>
<td>$3.58 \pm 0.10^b$</td>
<td>$4.57 \pm 0.12^c$</td>
</tr>
<tr>
<td>$V_a/100g \text{ BW}$</td>
<td>$2.10 \pm 0.05$</td>
<td>$2.07 \pm 0.08$</td>
<td>$2.12 \pm 0.10$</td>
<td>$2.09 \pm 0.09$</td>
</tr>
<tr>
<td>$V_d$</td>
<td>$2.33 \pm 0.04^a$</td>
<td>$1.88 \pm 0.06^b$</td>
<td>$1.85 \pm 0.09^b$</td>
<td>$2.36 \pm 0.07^a$</td>
</tr>
<tr>
<td>$V_d/100g \text{ BW}$</td>
<td>$1.02 \pm 0.03$</td>
<td>$1.11 \pm 0.07$</td>
<td>$1.07 \pm 0.05$</td>
<td>$1.09 \pm 0.06$</td>
</tr>
<tr>
<td>$V_w$</td>
<td>$1.33 \pm 0.05^a$</td>
<td>$1.04 \pm 0.06^b$</td>
<td>$1.46 \pm 0.07^c$</td>
<td>$1.59 \pm 0.07^c$</td>
</tr>
<tr>
<td>$V_w/100g \text{ BW}$</td>
<td>$0.58 \pm 0.02^a$</td>
<td>$0.61 \pm 0.02^a$</td>
<td>$0.85 \pm 0.04^b$</td>
<td>$0.73 \pm 0.03^c$</td>
</tr>
</tbody>
</table>

Absolute volume of alveolar air = $V_a$; of alveolar duct air = $V_d$; of alveolar wall = $V_w$.

Values are mean $\pm$ SEM.

$^a,^b,^c$ Means in each horizontal row without a common superscript are significantly different ($P<0.05$) by analysis of variance and SNK multiple range test.
The qualitative morphometric data obtained by point and intercept counts of the light microscopic tissue sections are shown in Table VIII. The mean linear intercept $L_m$, a morphometric estimate of the mean size of peripheral airspaces at maximum inflation, in the lungs of the untreated diabetic rats was 82% and 77% of the normal control and undernourished control values, respectively. The size of alveolar ducts, as assessed by the mean chord length, in the lungs of the untreated diabetic rats was 91% and 87% of the normal control and undernourished control values, respectively. The size of alveoli, as assessed by the mean chord length, was 80% and 75% of the normal control and undernourished control values, respectively, and very close to the changes in $L_m$. The surface area of alveoli in the lungs of untreated diabetic animals was 83% of the values in the normal control animals and 121% of that in the undernourished control animals. However, in the lungs of the untreated diabetic animals the alveolar surface area normalized for body weight was 115% of the normal control values and 125% of values in the undernourished control animals. The surface area:volume ratio in the untreated diabetic animals was 109% and 148% of values in the normal control and undernourished control animals, respectively, and indicated a change in the internal
complexity of the lungs often associated with alveolar proliferation. In this study, the $V_a:I_a^3$ ratio was used as an estimate of the number of alveoli per lung ($N_a$). The total number of alveoli in the lungs of untreated diabetic animals was 107% and 166% of the normal control and undernourished control values, respectively. However, the number of alveoli per unit of lung volume in the untreated diabetic animals was 136% of the normal control values and 155% of values in the undernourished control animals. Thus it was apparent that experimental diabetes produced smaller alveoli irrespective of the change in body size whereas the number of alveoli was increased particularly when corrected for the altered body size. Insulin treatment of the diabetic animals failed to completely reverse the changes in sizes of alveoli and alveolar ducts to normal control values.
Table VIII: Experimental Diabetes-induced Changes in Morphometric Aspects of Lung Growth

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Normal Control</th>
<th>Undernourished Control</th>
<th>Untreated Diabetic</th>
<th>Insulin-treated Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Lm (um)</td>
<td>114.20 ± 1.76a</td>
<td>120.85 ± 1.92b</td>
<td>93.19 ± 2.01c</td>
<td>103.66 ± 1.97d</td>
</tr>
<tr>
<td>Lg (um)</td>
<td>80.73 ± 0.92a</td>
<td>83.85 ± 0.99b</td>
<td>73.31 ± 1.05c</td>
<td>77.22 ± 1.09d</td>
</tr>
<tr>
<td>Ls (um)</td>
<td>40.43 ± 0.56a</td>
<td>42.39 ± 0.63b</td>
<td>35.45 ± 0.69c</td>
<td>37.47 ± 0.69d</td>
</tr>
<tr>
<td>Sw (m²)</td>
<td>0.425 ± 0.012a</td>
<td>0.290 ± 0.014b</td>
<td>0.351 ± 0.020c</td>
<td>0.391 ± 0.017d</td>
</tr>
<tr>
<td>Sw/BWx10⁴</td>
<td>18.55 ± 0.21a</td>
<td>17.06 ± 0.16b</td>
<td>21.41 ± 0.29c</td>
<td>19.01 ± 0.23d</td>
</tr>
<tr>
<td>(m²/g)</td>
<td>47.77 ± 1.02a</td>
<td>35.17 ± 1.15b</td>
<td>52.09 ± 1.09c</td>
<td>49.92 ± 1.11d</td>
</tr>
<tr>
<td>Svw (cm⁻¹)</td>
<td>7.29 ± 0.18a</td>
<td>4.71 ± 0.12b</td>
<td>7.83 ± 0.21c</td>
<td>7.99 ± 0.19c</td>
</tr>
<tr>
<td>Na (x10⁻⁷)</td>
<td>77.84 ± 1.52a</td>
<td>68.21 ± 1.17b</td>
<td>105.85 ± 2.11c</td>
<td>97.69 ± 1.65d</td>
</tr>
<tr>
<td>(cm⁻³)</td>
<td>77.84 ± 1.52a</td>
<td>68.21 ± 1.17b</td>
<td>105.85 ± 2.11c</td>
<td>97.69 ± 1.65d</td>
</tr>
</tbody>
</table>

Lm = Mean linear intercept; Lg = Mean chord length of alveolar duct; 
Ls = Mean chord length of alveoli; 
Sw = Alveolar surface area; Svw = Surface area:volume ratio; 
Na = Total number of alveoli; 
Na/Vl = Alveolar number per unit volume.

Values are mean ± SEM.

Means in each horizontal row without a common superscript are significantly different (P<0.05) by analysis of variance and SNK multiple range test.
E. Changes in Pulmonary Surfactant Relative to Lung Growth

As shown in Table IX, the lung total phospholipid content in the untreated diabetic rats was 69% of normal control values but 117% of the values in undernourished control animals. The total contents of airway and tissue disaturated phosphatidylcholine (DSPC) were similarly altered. However, the airway DSPC content relative to total lung DSPC and to the alveolar surface area of the lungs was not different between the experimental groups of animals, and thus indicated that the experimental diabetes-induced changes in the pulmonary surfactant material were compatible with the altered body and lung growth.
**Table IX:** Lung Disaturated Phosphatidylcholine (DSPC) in Experimental Diabetes.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Normal Control</th>
<th>Undernourished Control</th>
<th>Untreated Diabetic</th>
<th>Insulin-treated Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>PL_T (mg)</td>
<td>18.96 ± 0.99^a</td>
<td>11.25 ± 1.02^b</td>
<td>13.17 ± 1.22^c</td>
<td>18.65 ± 1.16^A</td>
</tr>
<tr>
<td>DSPC_a (mg)</td>
<td>1.25 ± 0.06^a</td>
<td>0.72 ± 0.02^b</td>
<td>0.86 ± 0.04^c</td>
<td>1.10 ± 0.08^A</td>
</tr>
<tr>
<td>DSPC_t (mg)</td>
<td>6.20 ± 0.39^a</td>
<td>3.92 ± 0.22^b</td>
<td>4.76 ± 0.27^c</td>
<td>5.98 ± 0.31^A</td>
</tr>
<tr>
<td>DSPC_a [%]</td>
<td>16.59 ± 0.75</td>
<td>15.98 ± 0.83</td>
<td>16.66 ± 0.91</td>
<td>16.73 ± 0.89</td>
</tr>
<tr>
<td>DSPC_a/S_w (mg/m^2)</td>
<td>2.56 ± 0.15</td>
<td>2.52 ± 0.15</td>
<td>2.49 ± 0.19</td>
<td>2.48 ± 0.22</td>
</tr>
</tbody>
</table>

PL_T = total phospholipids/Lung; DSPC_a = Airway DSPC/Lung; DSPC_t = Tissue DSPC/Lung;

DSPC_a [%] = [Airway DSPC/Total DSPC] x 100;

DSPC_a/S_w = Airway DSPC per Alveolar surface area

Values are mean ± SEM.

^a,b^ Means in each horizontal row without a common superscript are significantly different (P<0.05) by analysis of variance and SNK multiple range test.
F. In vivo Metabolic Changes

In order to study the in vivo synthetic activities of lung connective tissue by measurements of incorporation of [\(^{14}\)C]proline and the production of [\(^{14}\)C]hydroxyproline, it was necessary to establish a method of adequately separating the hydroxyproline from proline. Figure 9 shows a typical elution pattern of standard radiolabeled hydroxyproline and proline by the Dowex 50-X12 chromatography columns that were set up for this purpose in the present study. The average recovery of the standards from each batch of resin was 96%, and essentially all of the radioactivity in the lung samples was accounted for in the hydroxyproline and proline peaks. Figure 10 shows the changes in serum proline specific activity after an intraperitoneal injection of tracer amounts of [\(^{14}\)C]proline. There was a rapid decrease during the first 45 minutes, followed by a gradual decline to zero activity at 12 to 24 hours, and indicate the rapid equilibration between serum and tissue pools of proline. Radioactive hydroxyproline was not detected in the serum samples. The specific activities of serum proline were significantly lower in the diabetic rats than in the control animals, although the serum proline concentrations were not significantly different between the experimental groups of animals.

Since the specific activity of the labeled precursor influences the radioactivity incorporated into tissue proteins, it was necessary to correct for the differences in serum proline specific activities between
the experimental groups of animals. The ratios of the areas contained
under the curve of serum proline specific activities in normal control
rats to those of the other groups of experimental animals were calculated
(Table X) and used as correction factors in the estimation of in vivo
synthetic activities of the lung connective tissues.

Other investigators (132,133) have used a similar correction factor.
Similar proline specific activity ratios were evident in the TCA-soluble
extracts of lung homogenates at 12 and 24 hours after the injection of
$^{14}$C]proline. Thus the estimated in vivo $^{14}$C]proline incorporation
and $^{14}$C]hydroxyproline production values in lungs of undernourished
control, untreated diabetic and insulin-treated diabetic animals were
multiplied by 1.09, 1.50 and 1.33, respectively.
Figure 9: Separation of hydroxyproline and proline by Dowex 50-X12 column chromatography. Standard $[^3]$H]hydroxyproline (0.40 uCi) and $[^14]$C]proline (0.48 uCi) were applied to the column and eluted with 1 N HCl as described in Experimental Procedures. Radioactivity in each fraction was determined by double-channel counting.
Figure 10: Specific activity of free proline in sera of the experimental animals at various times after the injection of $^{14}$Cproline. Each point represents the mean ± SEM of three experiments, each of which involved the pooled sera of two animals. Mean ± SEM serum levels of proline (umole/mL) were 0.25 ± 0.08 in the normal control, 0.28 ± 0.10 in the undernourished control, 0.22 ± 0.09 in the untreated diabetic and 0.30 ± 0.11 in the insulin-treated diabetic animals.
<table>
<thead>
<tr>
<th>Proline Specific Activity Ratios*</th>
<th>Serum</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 hrs.</td>
<td>24 hrs.</td>
</tr>
<tr>
<td>Normal Control/Undernourished Control</td>
<td>1.09</td>
<td>1.13</td>
</tr>
<tr>
<td>Normal Control/Untreated Diabetic</td>
<td>1.50</td>
<td>1.51</td>
</tr>
<tr>
<td>Normal Control/Insulin-treated Diabetic</td>
<td>1.33</td>
<td>1.35</td>
</tr>
</tbody>
</table>

*Proline specific activity ratios in serum were calculated from the areas under the curves of specific activity of free proline after injection of the radiolabel (Fig. 10), while those of the TCA-soluble extracts of lung homogenates (the tissue pool) were estimated at the times indicated.
Table XI shows the changes in total $[^{14}\text{C}]$proline incorporation and in total $[^{14}\text{C}]$hydroxyproline production in PBS-soluble lung fractions at 24 hours after the injection of $[^{14}\text{C}]$proline, and indicated that total $[^{14}\text{C}]$proline incorporation into lungs of untreated diabetic animals was reduced compared to that in lungs of normal controls but was higher than that in the undernourished controls. The incorporation into lungs of the insulin-treated diabetic animals was greater than that in the normal controls. However, the proportion of total incorporation that constituted $[^{14}\text{C}]$hydroxyproline production was only slightly lower in the undernourished control animals, and thus suggested that the changes in $[^{14}\text{C}]$hydroxyproline production may not be due to altered hydroxylation of proline.
Table XI: Effects of Experimental Diabetes on $^{[14C]}$Proline incorporation and $^{[14C]}$Hydroxyproline production in Soluble Proteins of Lungs, 24 hours after injection of the radiolabeled proline.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>A. Total $^{[14C]}$OHPro (dpm x 10$^{-3}$/Lung)</th>
<th>B. Total $^{[14C]}$Pro Incorporated (dpm x 10$^{-3}$/Lung)</th>
<th>A/B x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>2.99 ± 0.20$^a$</td>
<td>53.64 ± 1.87$^a$</td>
<td>5.65</td>
</tr>
<tr>
<td>Undernourished</td>
<td>1.51 ± 0.13$^b$</td>
<td>30.76 ± 1.22$^b$</td>
<td>4.97</td>
</tr>
<tr>
<td>Diabetic</td>
<td>2.48 ± 0.19$^c$</td>
<td>45.52 ± 1.63$^c$</td>
<td>5.54</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>3.25 ± 0.27$^d$</td>
<td>55.82 ± 1.91$^d$</td>
<td>5.91</td>
</tr>
</tbody>
</table>

Values are means ± SEM; 5 animals per experimental group.  
a, b, c, d For each parameter measured, means without a common superscript are significantly different (P<0.05) by analysis of variance and SNK multiple range test.
Table XII shows the activities of $[^{14}C]$hydroxyproline in PBS-soluble and PBS-insoluble fractions of lung samples after injection of $[^{14}C]$proline. At 6 hours, the activities of soluble $[^{14}C]$hydroxyproline per mg DNA and per lung in the untreated diabetic animals were significantly reduced compared to normal controls but increased compared to the undernourished controls, whereas only the activities of insoluble $[^{14}C]$hydroxyproline per mg DNA in the untreated diabetic animals were similar to those of normal controls but increased compared to the undernourished control values. The activities of soluble and insoluble $[^{14}C]$hydroxyproline in the insulin-treated diabetic rats were higher than those in the normal controls. When the total activities of soluble and of insoluble $[^{14}C]$hydroxyproline were expressed as percent of their sum, approximately 10.4% of the activities was present in the insoluble $[^{14}C]$hydroxyproline of lungs in the untreated diabetic animals compared to 9.2%, 7.7% and 9.8% in the normal control, undernourished control and insulin-treated diabetic animals, respectively. At 24 hours after the injection of $[^{14}C]$proline, the activities of soluble $[^{14}C]$hydroxyproline per mg DNA and per lung in the untreated diabetic animals were lower compared to normal control values but increased compared to the undernourished control values. The activities of the insoluble $[^{14}C]$hydroxyproline per mg lung DNA in the untreated diabetic animals were similar to those of the normal controls.
but increased compared to the undernourished control values. However, the total activities of the insoluble $[^{14}\text{C}]$hydroxyproline in the untreated diabetic rat lungs were reduced compared to the normal control values, but represented 60.9% of the total of soluble and insoluble incorporations compared to 58.1%, 47.8% and 59.2% in the normal control, undernourished control and insulin-treated diabetic animals, respectively. These results thus indicate that the cellular incorporation of $[^{14}\text{C}]$proline into lung $[^{14}\text{C}]$hydroxyproline was increased in experimental diabetes when compared to the values of undernourished weight-matched control animals, but was relatively stable when compared to the values of normal control animals. However, the conversion of soluble to insoluble $[^{14}\text{C}]$hydroxyproline was increased, independent of body growth.
Table XII: Experimental Diabetes-induced Changes in Activities of $[^{14}\text{C}]$Hydroxyproline in Soluble and Insoluble Fractions of Lung after Injection of $[^{14}\text{C}]$Proline.

<table>
<thead>
<tr>
<th>Hrs after $[^{14}\text{C}]$- Proline injection</th>
<th>Normal Control</th>
<th>Undernourished Control</th>
<th>Untreated Diabetic</th>
<th>Insulin-treated Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>n 6 SOLUBLE $[^{14}\text{C}]$OHPro:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dpm/mg DNA</td>
<td>3891 ± 153$^a$</td>
<td>2502 ± 199$^b$</td>
<td>3356 ± 222$^c$</td>
<td>4057 ± 207$^d$</td>
</tr>
<tr>
<td>dpm/Lung</td>
<td>18602 ± 511$^a$</td>
<td>9826 ± 342$^b$</td>
<td>12457 ± 496$^c$</td>
<td>20762 ± 695$^d$</td>
</tr>
<tr>
<td>% of total activity</td>
<td>[90.8]</td>
<td>[92.3]</td>
<td>[89.6]</td>
<td>[90.2]</td>
</tr>
<tr>
<td>n 6 INSOLUBLE $[^{14}\text{C}]$OHPro:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dpm/mg DNA</td>
<td>392 ± 25$^a$</td>
<td>211 ± 17$^b$</td>
<td>381 ± 30$^a$</td>
<td>455 ± 27$^c$</td>
</tr>
<tr>
<td>dpm/Lung</td>
<td>1879 ± 119$^a$</td>
<td>835 ± 99$^b$</td>
<td>1442 ± 123$^c$</td>
<td>2350 ± 134$^d$</td>
</tr>
<tr>
<td>% of total activity</td>
<td>[9.2]</td>
<td>[7.7]</td>
<td>[10.4]</td>
<td>[9.8]</td>
</tr>
<tr>
<td>n 24 SOLUBLE $[^{14}\text{C}]$OHPro:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dpm/mg DNA</td>
<td>701 ± 39$^a$</td>
<td>440 ± 20$^b$</td>
<td>625 ± 18$^c$</td>
<td>781 ± 59$^d$</td>
</tr>
<tr>
<td>dpm/Lung</td>
<td>3368 ± 215$^a$</td>
<td>1671 ± 173$^b$</td>
<td>2313 ± 196$^c$</td>
<td>374 ± 236$^d$</td>
</tr>
<tr>
<td>% of total activity</td>
<td>[41.9]</td>
<td>[52.2]</td>
<td>[38.1]</td>
<td>[40.8]</td>
</tr>
<tr>
<td>n 24 INSOLUBLE $[^{14}\text{C}]$OHPro:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dpm/mg DNA</td>
<td>972 ± 77$^a$</td>
<td>415 ± 52$^b$</td>
<td>968 ± 15$^a$</td>
<td>1057 ± 81$^c$</td>
</tr>
<tr>
<td>dpm/Lung</td>
<td>4653 ± 266$^a$</td>
<td>1535 ± 180$^b$</td>
<td>3603 ± 214$^c$</td>
<td>5399 ± 273$^d$</td>
</tr>
<tr>
<td>% of total activity</td>
<td>[58.1]</td>
<td>[47.8]</td>
<td>[61.9]</td>
<td>[59.2]</td>
</tr>
</tbody>
</table>

Values are means ± SEM

$^a,b,c,d$ In each horizontal row, means without a common superscript are significantly different ($P \leq 0.05$) by analysis of variances and SNK multiple range test.
Hydroxyproline is exclusively a connective tissue amino acid found mostly in collagen but also present in elastin. Thus the changes observed in hydroxyproline contents and synthetic activities may represent alterations in collagen and/or elastin. As shown in Table XIII, the activities of lung collagen \[^{14}\text{C}]\text{hydroxyproline per mg lung DNA in the untreated diabetic rats were similar to those of the normal control animals but were 2-fold higher than the undernourished control values. However, the total activities were 80\% and 211\% of normal control and undernourished control values, respectively. The activities of lung \[^{14}\text{C}]\text{elastin per mg DNA in the untreated diabetic animals were unchanged compared to those of the normal control animals but were 3-fold higher than the undernourished control values. However, the total activities were 76\% and 150\% of the values in the normal control and undernourished control animals, respectively. Insulin-treated diabetic animals had similar activities of \[^{14}\text{C}]\text{elastin per mg DNA as the normal control animals but the total synthetic activities of collagen and elastin were increased. These results indicated that the experimental diabetes-induced reduction in total collagen and elastin synthesis may be a reflection of the reduced cell number in the lungs.}

The specific activities (dpm/mg DNA) and total activities (dpm/lung) of DNA were similar in the lungs of undernourished control and untreated diabetic rats but these were 60\% of normal control values. The activities of in the insulin-treated diabetic animals were higher than those of the normal control animals.
Table XIII: Experimental Diabetes-induced Changes in 24-hour Synthetic Activities of DNA, Collagen and Elastin in the lungs.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Normal Control</th>
<th>Undernourished Control</th>
<th>Untreated Diabetic</th>
<th>Insulin-treated Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>DNA:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dpm x 10^{-3}/mg</td>
<td>6.35 ± 0.31^a</td>
<td>5.21 ± 0.33^b</td>
<td>4.99 ± 0.52^b</td>
<td>6.98 ± 0.29^c</td>
</tr>
<tr>
<td>dpm x 10^{-3}/Lung</td>
<td>31.59 ± 1.36^a</td>
<td>18.04 ± 1.21^b</td>
<td>17.92 ± 1.63^b</td>
<td>35.61 ± 1.42^c</td>
</tr>
<tr>
<td>COLLAGEN:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[^{14}C]OHPro (dpm/mg DNA)</td>
<td>838 ± 68^a</td>
<td>395 ± 26^b</td>
<td>821 ± 90^a</td>
<td>991 ± 71^c</td>
</tr>
<tr>
<td>[^{14}C]OHPro (dpm/Lung)</td>
<td>4009 ± 221^a</td>
<td>1518 ± 105^b</td>
<td>3210 ± 264^c</td>
<td>4775 ± 259^d</td>
</tr>
<tr>
<td>ELASTIN:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[^{14}C]Elastin (dpm/mg DNA)</td>
<td>59 ± 5^a</td>
<td>17 ± 1^b</td>
<td>55 ± 6^a</td>
<td>63 ± 10^a</td>
</tr>
<tr>
<td>[^{14}C]Elastin (dpm/Lung)</td>
<td>274 ± 16^a</td>
<td>139 ± 10^b</td>
<td>209 ± 20^c</td>
<td>315 ± 25^d</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

a,b,c,d In each horizontal row, means without a common superscript are significantly different (P<0.05) by analysis of variance and SNK multiple range test.
Table XIV shows that the cellular and total activities of the adenylate cyclase cytosolic modulator and of cyclic AMP content were increased in the lungs of the untreated diabetic animals when compared to those of the normal control animals but were lower than those of the undernourished control animals. Lung calmodulin activity and the total content were reduced in the untreated diabetic animals when compared to the normal control values but were increased compared to the values of the undernourished control animals. However, there were no significant differences between the experimental groups with regards to cellular calmodulin content. Relative to the normal control animals, the insulin-treated diabetic rats had lower adenylate cyclase cytosolic modulator activity and cyclic AMP content but higher calmodulin content and activity.
Table XIV: Lung Adenylate Cyclase Cytosolic Modulator, Calmodulin and Cyclic AMP in Experimental Diabetes.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Normal Control</th>
<th>Undernourished Control</th>
<th>Untreated Diabetic</th>
<th>Insulin-treated Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
</tr>
<tr>
<td><strong>Adenylate Cyclase Activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosolic Modulator (Units/Lung x 10^{-2})</td>
<td>38.6 ± 0.2(^a)</td>
<td>57.5 ± 0.3(^b)</td>
<td>40.2 ± 0.3(^c)</td>
<td>35.3 ± 0.5(^d)</td>
</tr>
<tr>
<td>(Units/mg DNA)</td>
<td>799 ± 72(^a)</td>
<td>1505 ± 101(^b)</td>
<td>968 ± 83(^c)</td>
<td>694 ± 64(^d)</td>
</tr>
<tr>
<td><strong>Calmodulin Activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Units/Lung x 10^{-2})</td>
<td>57.5 ± 2.3(^a)</td>
<td>29.7 ± 1.9(^b)</td>
<td>34.8 ± 2.2(^c)</td>
<td>69.1 ± 2.6(^d)</td>
</tr>
<tr>
<td>(Units/mg DNA)</td>
<td>1190 ± 85(^a)</td>
<td>765 ± 62(^b)</td>
<td>915 ± 69(^c)</td>
<td>1352 ± 101(^d)</td>
</tr>
<tr>
<td><strong>Calmodulin Content</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ug/Lung)</td>
<td>26.25 ± 1.58(^a)</td>
<td>19.70 ± 0.99(^b)</td>
<td>23.66 ± 1.33(^c)</td>
<td>29.99 ± 1.62(^d)</td>
</tr>
<tr>
<td>(ug/mg DNA)</td>
<td>5.53 ± 0.59</td>
<td>5.16 ± 0.25</td>
<td>6.01 ± 0.72</td>
<td>5.89 ± 0.51</td>
</tr>
<tr>
<td><strong>Cyclic AMP Content</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmole/Lung)</td>
<td>1.65 ± 0.23(^a)</td>
<td>3.01 ± 0.33(^b)</td>
<td>1.81 ± 0.25(^c)</td>
<td>1.05 ± 0.15(^d)</td>
</tr>
<tr>
<td>(nmole/mg DNA)</td>
<td>0.34 ± 0.09(^a)</td>
<td>0.78 ± 0.12(^b)</td>
<td>0.48 ± 0.10(^c)</td>
<td>0.29 ± 0.05(^a)</td>
</tr>
</tbody>
</table>

* One unit of adenylate cyclase cytosolic modulator activity or calmodulin activity was defined as the amount which produces 50% of the maximal activation of standard adenylate cyclase or cyclic AMP-phosphodiesterase, respectively.

Values are means ± SEM.

\(^a,b,c,d\) In each horizontal row, means without a common superscript are significantly different (P<0.05) by analysis of variance and SNK multiple range test.
G. In vitro Effects of the Soluble Lung Extracts

In order to determine the role of endogenous regulatory factors in the altered DNA and connective tissue synthetic activities observed in the lungs of the experimental animals, lung minces prepared from younger normal rats were incubated with soluble lung extracts derived from the experimental animals. The effects of these extracts (50 ug protein/mL) on $[^3]$H]thymidine incorporation are shown in Figure 11. The extracts reduced the basal rate of $[^3]$H]thymidine incorporation in the lung minces, and this inhibitory effect was more pronounced with lung extracts derived from the undernourished control and untreated diabetic animals although both were similar in magnitude. There were no significant differences between the effects of normal control and insulin-treated diabetic rat lung extracts. Figure 12 shows that the inhibitory effect of the lung extracts on $[^3]$H]thymidine incorporation was concentration-dependent. The lung extracts of undernourished control and untreated diabetic rats caused a 50% inhibition at a concentration of 50 ug protein/mL whereas a similar level of inhibition was produced at a concentration of 150 ug protein/mL by the lung extracts of normal control and insulin-treated diabetic animals.
Figure 11: Effect of soluble lung extracts on $[^3]H$thymidine incorporation by normal lung mince in culture. Normal lung mince was incubated in the presence of 50 ug protein of the extracts per mL of medium. Values are mean ± SEM, n = 5. Significant differences (P<0.05) by analysis of variance and SNK multiple range test are cited in the text.
SOURCES OF LUNG EXTRACTS ADDED TO MEDIUM

1 hr incubation

- Normal control
- Insulin-treated diabetic
- Undernourished control
- Untreated diabetic

2 hrs incubation

BASAL

(3H) Thymidine incorporation
(dpm x 10^-3/μg DNA)
Figure 12: Concentration-dependent effects of soluble lung extracts on \([ ^3 \text{H} \text{]thymidine incorporation by normal lung mince in culture.}\]

Extracts derived from
Normal control (Δ),
Undernourished control (○),
Untreated diabetic (●) and
Insulin-treated diabetic (▲) animals were added at varying protein concentrations to normal lung mince in culture medium and incubated for two 2 hours.
Values are mean ± SEM (n = 5)
and are expressed as % of basal incorporation (see Figure 11).
Significant differences (P<0.05) by analysis of variance and SNK multiple range test are cited in the text.
(\(^3\)H) Thymidine incorporation (% of basal) vs. 
\[\mu g \text{ protein of lung extracts/ml of medium}\]
The effect of the lung extracts (150 ug protein/mL) on $[^{14}\text{C}]$hydroxyproline and $[^{14}\text{C}]$elastin production by the lung explant cultures is shown in Figure 13. The extracts suppressed the basal rate of soluble and polymeric collagen $[^{14}\text{C}]$hydroxyproline production and of $[^{14}\text{C}]$elastin production per mg DNA of the lung minces, and this inhibitory effect was 2-3 times greater with extracts of undernourished control animals compared to those of the other experimental animals. Although the inhibitory effects of extracts of the untreated diabetic animals on $[^{14}\text{C}]$hydroxyproline production were similar in magnitude to those of the normal controls, they were higher compared to those of the insulin-treated diabetic animals. However, there were no significant differences in inhibitory effects on $[^{14}\text{C}]$elastin production by extracts of normal control, untreated and insulin-treated diabetic animals. Figure 14 shows that the inhibitory effect of the lung extracts on hydroxyproline and connective tissue production was also concentration-dependent. As with $[^{3}\text{H}]$thymidine incorporation, the lung extracts of undernourished control animals produced a 50% inhibition of soluble and polymeric collagen $[^{14}\text{C}]$hydroxyproline production and of $[^{14}\text{C}]$elastin production at a concentration of 50 ug protein/mL. The extracts of untreated diabetic and normal control animals produced 50% inhibition of $[^{14}\text{C}]$hydroxyproline production at concentrations of about 150 ug protein/mL whereas those of insulin-treated diabetic animals caused similar level of inhibitions at about 200 ug protein/mL. Inhibitions of $[^{14}\text{C}]$elastin production by the lung extracts of normal control, untreated and insulin-treated diabetic rats were 50% only at concentrations of 250 ug protein/mL.
Figure 13: Effect of soluble lung extracts on soluble and collagen \([^{14}\text{C}]\text{hydroxyproline}\) and \([^{14}\text{C}]\text{elastin}\) production by normal lung mince in culture. The lung mince was incubated in the absence (■) or presence of extracts (150 \(\mu\text{g/mL}\)) derived from:
- Normal control (△),
- Undernourished control (○),
- Untreated diabetic (●) and
- Insulin-treated diabetic (▲) animals.

Values are mean \(±\) SEM, \(n = 5\). Significant differences (\(P<0.05\)) by analysis of variance and SNK multiple range test are cited in the text.

Rates of \([^{14}\text{C}]\text{hydroxyproline}\) and \([^{14}\text{C}]\text{elastin}\) production were linear up to 4 hour incubation.
(14C) Elastin production
(dpm × 10^{-2}/mg DNA)

Collagen (14C)
Hydroxyproline production
(dpm × 10^{-3}/mg DNA)

Soluble (14C) Hydroxyproline production
(dpm × 10^{-3}/mg DNA)

Hours of incubation
Figure 14: Concentration-dependent effect of soluble lung extracts on soluble and collagen $[^{14}C]$hydroxyproline and $[^{14}C]$elastin production by normal lung mince in culture. Extracts derived from
Normal control ($\triangle$),
Undernourished control (○),
Untreated diabetic (●) and
Insulin-treated diabetic (▲) animals

were added at varying protein concentrations to the lung mince in culture medium and incubated for 3 hours.
Values are mean ± SEM (n = 5) and are expressed as
% of the basal metabolic values (see Figure 13).
Significant differences (P<0.05) by analysis of variance and SNK multiple range test are cited in the text.
The diagrams depict the effects of varying concentrations of lung extracts on the production of soluble (\(^{14}\text{C}\)) OHPF and collagen production. The graphs show a decrease in production as the concentration of lung extracts increases, with different symbols representing different conditions or treatments. The x-axis represents the concentration of protein in lung extracts (\(\mu\text{g}\)) per ml of medium, while the y-axis shows the production levels as a percentage of basal production.
As shown in Figure 15, the suppressive activity of the lung extracts on $[^3]H$thymidine incorporation was reduced by prior boiling of the extracts at $90^\circ C$ for 5 min. Suppressive activities of extracts of normal control and insulin-treated diabetic animals changed from 51% and 53% of basal $[^3]H$thymidine incorporation to 85% and 81%, respectively. Interestingly, those of the undernourished control and untreated diabetic rats changed from 32% to 83% and 34% to 71%, respectively. When the heat-treated lung extracts of the experimental animals were further treated in batch with DEAE-cellulose containing 0.25 M NaCl, the suppressive activities of the eluents increased but were still lower than the original activities. Trypsinization of the lung extracts abolished completely their suppressive effects on $[^3]H$thymidine incorporation. Similarly, boiling reduced the suppressive activities of the lung extracts on $[^14]C$hydroxyproline productions from 60% to 84% of basal production rate with extracts of normal controls, from 34% to 83% with extracts of undernourished controls, from 56% to 90% with extracts of untreated diabetic animals and 68% to 87% with extracts of insulin-treated diabetic animals (Figure 16). Treatment of the boiled extracts with the DEAE-cellulose/NaCl mixture did not produce any further change in the suppressive activities of the extracts. Trypsinization abolished the inhibitory effect of the extracts on $[^14]C$hydroxyproline. These findings thus suggested the heterogenous and protein nature of the endogenous regulatory factors.
Figure 15: Effects of boiling and subsequent treatment with DEAE-cellulose containing 0.25 M NaCl and of trypsin on the suppressive activities of the soluble extracts with regards to $[^3H]$thymidine incorporation.

Aliquots of the extracts containing 150 ug protein/mL were treated accordingly as described in Experimental Procedures. Trypsinization of the extracts was performed by incubation for 15 min with 0.2 mg/mL of trypsin insolubilized on sepharose beads (Sigma) and terminated by centrifugation and separation of the enzyme-coated beads. All treated extracts were made to original volume, filtered and then added to the lung mince cultures and incubated for 2 hours. Values are mean ± SEM (n = 6) and are expressed as % of basal incorporation (see Figure 11). Significant differences (P<0.05) by analysis of variance and SNK multiple range test are cited in the text.
SOURCES OF LUNG EXTRACTS

- Normal control
- Insulin-treated diabetic
- Undernourished control
- Untreated diabetic

Bar graph showing thymidine incorporation (% of basal) for different sources of lung extracts with various treatments:

- None
- 90°C, 5 min
- 90°C, 5 min + DEAE
- Trypsinization
Figure 16: Effects of heat and subsequent batch treatment with DEAE-cellulose containing 0.25 M NaCl and of trypsin on the suppressive activities of the soluble lung extracts with regards to $[^{14}C]$hydroxyproline production. Amounts of extracts and the treatments were similar to those described in Figure 15. Normal lung mince was incubated in the presence of the extracts for 3 hours. Values are mean $\pm$ SEM ($n = 5$) and are expressed as

$\%$ of basal $[^{14}C]$hydroxyproline production

$(10.23 \pm 0.99 \text{ dpm} \times 10^{-3} / \text{mg DNA})$.

$[^{14}C]$hydroxyproline represented total activity in the lung mince and thus did not differentiate between collagen and elastin. Significant differences ($P<0.05$) by analysis of variance and SNK multiple range test are cited in the text.
### SOURCES OF LUNG EXTRACTS

<table>
<thead>
<tr>
<th>Source</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>None</td>
</tr>
<tr>
<td>Insulin-treated diabetic</td>
<td>90°C, 5 min</td>
</tr>
<tr>
<td>Undernourished control</td>
<td>90°C, 5 min + DEAE</td>
</tr>
<tr>
<td>Untreated diabetic</td>
<td>Trypsinization</td>
</tr>
</tbody>
</table>

**HYDROXYPROLINE PRODUCTION (% OF BASEL)**

- **None**
- **90°C, 5 min**
- **90°C, 5 min + DEAE**
- **Trypsinization**
Table XV shows that there was a dramatic reduction in cyclic AMP contents of the lung minces, from 452 to 79 pmole/mg DNA, at 1 to 4 hours of incubation. Addition of lung extracts to the incubation media caused an increase in the tissue cyclic AMP contents at 1 hour, with extracts derived from the undernourished control, untreated diabetic, normal control and insulin-treated diabetic animals producing 199%, 173%, 134% and 113% of the basal values, respectively. These effects of the lung extracts were also concentration-dependent and decreased at 4 hours of incubation, although still maintaining the differences observed at 1 hour. It was thus evident that the lung extracts produced increase in lung tissue cyclic AMP levels as well as the suppression of cellular proliferation and connective tissue production.
Table XV: Effects of the Soluble Lung Extracts on cyclic AMP contents of Normal Lung Tissues incubated for 1 to 4 hours in DMEM at 37°C.

<table>
<thead>
<tr>
<th>Lung Extracts</th>
<th>Hours of incubation</th>
<th>Cyclic AMP (pmoles x 10^{-2}/mg DNA of Lung)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Control</td>
<td>4.52 ± 0.36</td>
<td>0.79 ± 0.02</td>
</tr>
<tr>
<td>50 ug/ml</td>
<td>6.06 ± 0.50</td>
<td>1.02 ± 0.05</td>
</tr>
<tr>
<td>100 ug/ml</td>
<td>8.59 ± 0.53</td>
<td>1.56 ± 0.09</td>
</tr>
<tr>
<td>Undernourished Control</td>
<td>9.01 ± 0.46</td>
<td>1.82 ± 0.10</td>
</tr>
<tr>
<td>50 ug/ml</td>
<td>13.15 ± 0.59</td>
<td>2.30 ± 0.12</td>
</tr>
<tr>
<td>100 ug/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated Diabetic</td>
<td>7.84 ± 0.55</td>
<td>1.35 ± 0.07</td>
</tr>
<tr>
<td>50 ug/ml</td>
<td>10.19 ± 0.62</td>
<td>1.99 ± 0.12</td>
</tr>
<tr>
<td>100 ug/ml</td>
<td>5.13 ± 0.41</td>
<td>0.88 ± 0.07</td>
</tr>
<tr>
<td>Insulin-treated Diabetic</td>
<td>7.69 ± 0.49</td>
<td>1.30 ± 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SDM of five independent experiments performed in triplicate.

n = 7 for each experimental groups.
Since lung cyclic AMP levels may be regulated by the adenylate cyclase cytosolic modulator and/or by calmodulin (58-60,110), it was necessary to determine the effects of lung fractions, enriched with these endogenous modulators, on $[^3H]$thymidine incorporation and $[^{14}C]$hydroxyproline production. Figure 17 shows a typical elution profile of the soluble lung extracts on a column of Sephadex G-75. Fractions of the eluents were assayed for adenylate cyclase cytosolic modulator and calmodulin activities, and the fractions were subsequently pooled as indicated to give four major fractions such that fraction II contained the adenylate cyclase cytosolic modulator activities and fraction III contained the calmodulin activities. As shown in Table XVI, only fractions II and III of the lung extracts significantly increased the cyclic AMP contents of the explants with concomitant suppression of $[^3H]$thymidine incorporation and $[^{14}C]$hydroxyproline production. The suppressive effects of the fractions on $[^3H]$thymidine incorporation were greater than the effects on $[^{14}C]$hydroxyproline production. Fractions II of lung extracts derived from the undernourished control rats were more potent in both suppressive activities compared to those derived from the other experimental groups of animals which were similar, although the effects of the untreated diabetic lung extracts on $[^{14}C]$hydroxyproline were less compared to those of the insulin-treated diabetic animals. Fractions III of the lung extracts of normal and undernourished control and insulin-treated diabetic animals were similar.
in their suppressive effects on $[^3\text{H}]$thymidine incorporation but lower than those of the untreated diabetic animals. However, with regards to the suppressive effects on $[^{14}\text{C}]$hydroxyproline production, fractions III of the lung extracts derived from the diabetic animals were less potent than those of the control animals. These results thus suggest that elevations in the cyclic AMP levels produced by the adenylate cyclase cytosolic modulator and calmodulin may be responsible for the suppressive activities of the lung extracts on $[^3\text{H}]$thymidine incorporation and $[^{14}\text{C}]$hydroxyproline production. However, the findings that fractions III of the untreated diabetic animals had greater suppressive activities on $[^3\text{H}]$thymidine incorporation but lower activities on $[^{14}\text{C}]$hydroxyproline production was interesting and warranted further studies.
Figure 17: Sephadex G-75 column chromatography of the soluble lung extracts. Samples of the extracts (3.5 mg protein) were chromatographed on the column as described in Experimental Procedures. Extracts from all the experimental groups chromatographed in a similar fashion. For the assay of adenylate cyclase cytosolic modulator (AC-CM) activity, 50 - 200 µL of the eluted fractions were used to activate adenylate cyclase in a lung membrane preparation. For the assay of calmodulin (CaM) activity, 10 - 75 µL of the eluted fractions were used to activate a standard calmodulin-deficient phosphodiesterase. One unit of activity was defined as the amount which produced 50% of the maximal activation of the enzyme. Fractions were pooled as indicated and applied to the lung mince cultures.
Table XVI: Effects of Sephadex G75-Fractionated Lung Extracts on cyclic AMP contents, [\(^{3}\)H]Thymidine Incorporation and [\(^{14}\)C]Hydroxyproline Production of Normal Lung Explants incubated for 4 hours in DMEM at 37°C.

<table>
<thead>
<tr>
<th>Fractions of Lung Extracts (µg protein/ml)</th>
<th>Cyclic AMP (pmole/mg DNA)</th>
<th>[(^{3})H]Thd Incorporation (dpm x 10(^{-3})/mg DNA)</th>
<th>[(^{14})C]OHPro Production (dpm x 10(^{-3})/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>85 ± 2</td>
<td>13.73 ± 0.99</td>
<td>17.21 ± 1.27</td>
</tr>
<tr>
<td>Normal Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I 125</td>
<td>79 ± 7</td>
<td>12.91 ± 0.91</td>
<td>18.67 ± 1.36</td>
</tr>
<tr>
<td>II 89</td>
<td>165 ± 5</td>
<td>8.25 ± 0.75*</td>
<td>12.05 ± 0.79*</td>
</tr>
<tr>
<td>III 50</td>
<td>124 ± 3</td>
<td>7.90 ± 0.51*</td>
<td>13.14 ± 0.99*</td>
</tr>
<tr>
<td>IV 35</td>
<td>91 ± 5</td>
<td>15.38 ± 1.08</td>
<td>16.01 ± 1.05</td>
</tr>
<tr>
<td>Undernourished Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I 99</td>
<td>82 ± 6</td>
<td>13.00 ± 1.00</td>
<td>19.15 ± 1.51</td>
</tr>
<tr>
<td>II 132</td>
<td>203 ± 7</td>
<td>5.39 ± 0.29*</td>
<td>8.61 ± 0.50*</td>
</tr>
<tr>
<td>III 24</td>
<td>135 ± 3</td>
<td>7.18 ± 0.66*</td>
<td>12.05 ± 0.88*</td>
</tr>
<tr>
<td>IV 55</td>
<td>75 ± 10</td>
<td>14.50 ± 1.03</td>
<td>17.04 ± 1.19</td>
</tr>
<tr>
<td>Untreated Diabetic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I 123</td>
<td>90 ± 4</td>
<td>12.55 ± 1.13</td>
<td>18.93 ± 1.25</td>
</tr>
<tr>
<td>II 115</td>
<td>183 ± 5</td>
<td>7.96 ± 0.42*</td>
<td>11.36 ± 0.92*</td>
</tr>
<tr>
<td>III 39</td>
<td>199 ± 7</td>
<td>4.85 ± 0.20*</td>
<td>15.56 ± 1.11*</td>
</tr>
<tr>
<td>IV 22</td>
<td>81 ± 6</td>
<td>14.62 ± 1.30</td>
<td>16.88 ± 1.32</td>
</tr>
<tr>
<td>Insulin Treated Diabetic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I 141</td>
<td>90 ± 7</td>
<td>13.05 ± 1.11</td>
<td>19.00 ± 1.41</td>
</tr>
<tr>
<td>II 80</td>
<td>146 ± 4</td>
<td>8.63 ± 0.69*</td>
<td>13.01 ± 0.90*</td>
</tr>
<tr>
<td>III 47</td>
<td>151 ± 5</td>
<td>7.44 ± 0.33*</td>
<td>14.11 ± 1.05*</td>
</tr>
<tr>
<td>IV 31</td>
<td>88 ± 4</td>
<td>13.99 ± 1.20</td>
<td>16.82 ± 1.29</td>
</tr>
</tbody>
</table>

Values are means ± SEM of three independent experiments performed in triplicates (n=5 for each experimental group).

* Significantly different from basal (P<0.05) by analysis of variance and SNK multiple range test.
As shown in Figure 18, inclusion of trifluoperazine, a potent calmodulin inhibitor, in the explant culture medium containing fractions II of the lung extracts derived from the experimental animals did not alter the suppressive effects of the fractions on $[^3\text{H}]$thymidine incorporation and $[^1\text{C}]$hydroxyproline production. However, boiling of the lung extract fractions abolished the suppressive activities. These findings are consistent with our previous observations(58,110) that the adenylate cyclase cytosolic modulator is heat-labile and distinctively not calmodulin. Trifluoperazine increased the suppressive activities of fractions III of the lung extract on $[^3\text{H}]$thymidine incorporation but did not affect the activities on $[^1\text{C}]$hydroxyproline production. Boiling of the fractions did not alter their suppressive activities on $[^3\text{H}]$thymidine incorporation and $[^1\text{C}]$hydroxyproline production. It was thus apparent that although fractions III contained calmodulin activity, their suppressive effects on cellular proliferation and connective tissue production may not be due to calmodulin but to possibly a calmodulin inhibitor and/or a different factor altogether.
Figure 18: Effects of trifluoperazine and boiling on the suppressive activity of Sephadex G-75 column chromatography-derived soluble lung extracts. Pooled fractions (II and III; see Figure 17) of extracts from Normal control (open bars), Undernourished control (hatched bars), Untreated diabetic (solid bars) and Insulin-treated diabetic (dotted bars) animals were either untreated (A), mixed with 50 μM Trifluoperazine (B) or boiled (C) prior to addition to lung mince cultures. Protein concentrations are identical to those in Table XVI. [³H]thymidine incorporation and [¹⁴C]hydroxyproline was measured after 4 hours incubation. Values are mean + SEM (n = 7) and represent % of basal metabolic values (see Table XVI) Significant differences (P<0.05) by analysis of variance and SNK multiple range test are cited in the text.
Fraction III of the lung extracts were chromatographed on a calmodulin-specific affinity column comprised of fluphenazine covalently linked to Sepharose 6B, first without EGTA in the buffer such that only unbound proteins were eluted, and then with EGTA in the elution buffer such that the bound calmodulin was eluted. As shown in Figure 19, the unbound proteins (non-calmodulin) suppressed both $[^3]H$thymidine incorporation and $[^14]C$hydroxyproline production, while the bound proteins (calmodulin) enhanced $[^3]H$thymidine incorporation without any effect on $[^14]C$hydroxyproline production. When pooled fractions of the unbound proteins were further chromatographed on Sephadex G-75 columns (Figure 20), the eluted fractions contained a single peak of suppressive activity for $[^3]H$thymidine incorporation, at a position corresponding to apparent molecular weights of 17000-25000 daltons, and another peak of suppressive activity for $[^14]C$hydroxyproline production, at a position corresponding to apparent molecular weights of 13700-17000 daltons.
Figure 19: Fluphenazine-Sepharose affinity chromatography.

Aliquots (200 μL) of fractions III obtained by Sephadex G75 chromatography (see Figure 17) were combined and added to 400 μL of the column buffer. This sample was added to the column and eluted as described in Experimental Procedures. Fractions were pooled as indicated, lyophilized, re-suspended in culture medium and applied to the lung mince cultures.

[^3]H]thymidine incorporation and [14C]hydroxyproline production by the lung mince was measured after 4 hours incubation. Values represent the mean of separate determinations; individual values were within 10% of the mean values.

Data shown are for normal control lung fractions. Activity from the other experimental groups chromatographed similarly.
Figure 20: Sephadex G-75 column chromatography of Fluphenazine-Sepharose-derived fraction. 1.5 mg protein of Fraction IIIa (see Figure 19) was lyophilized, re-suspended in PBS and applied onto the column. Chromatography was performed as described in Experimental Procedures. 200 μL of the eluted fractions was added to the lung mince culture. [³H]thymidine incorporation and [¹⁴C]hydroxyproline production are reported as % inhibition of basal activity shown in Table XVI. Values are mean of independent determinations; individual values were within 10% of the mean. The column was standardized with proteins of known molecular weight; large arrow shows the elution position of affinity-purified calmodulin. Data shown are for normal control lung fractions. Activity from the other experimental groups chromatographed similarly.
V. DISCUSSION

A. Body Growth in the Experimental Animals

This study, like many others (86,110-115,134,135), indicated that experimental diabetes caused a smaller gain in body weight compared to that in the normal control animals. Although increase in body weight with time is the most widely used measure of somatic growth, body weight may also have changed as a result of altered water and fat metabolism, without a concomitant change in lean body mass or skeletal dimensions. This was an important issue to consider since this study is particularly interested in lung growth, and somatic growth appears to influence lung growth (1,6,75,77). Diabetes mellitus is a carbohydrate metabolic disorder with attendant loss of body fat, protein and even cellular water (86). In fact, this disease has been described as cellular "starvation in the midst of plenty". Thus it was necessary, in the present study, to include a group of undernourished control animals weight-matched for the untreated diabetic animals.

Since the data indicated that the undernourished control and the untreated diabetic rats had similar skeletal dimensions as measured by nose-tail lengths, it is likely that somatic growth was similarly altered in the undernourished control and untreated diabetic animals. In fact, if the nose-tail lengths of the untreated diabetic and undernourished control animals are cubed to get a three-dimensional quantity of body size, body weight and nose-tail length in these experimental groups of
animals remain similarly altered. Hence, it appeared that the undernourished control animals afforded this study with a reasonable reference group for estimating specific diabetes-induced effects irrespective of somatic growth.

Insulin treatment of the diabetic animals restored the body weight and nose-tail length to within statistically insignificant values of the normal control. This may be due to the generalized anabolic effect of insulin on the organs and tissues of the body (86). However, it was observed that these animals still exhibited mild polyuria, glycosuria and hyperglycemia at the time of sacrifice. Thus the dose of insulin given to these animals may be sufficient for the normalization of body size but is not adequately controlling the disorder of carbohydrate metabolism. This may have resulted from the fact that the daily dose of insulin given to these rats was dependent solely on the daily measurements of body weight, urinary output and urine glucose; serum glucose was not determined until the time of sacrifice. How this may affect the measured parameters of lung growth and structure will be discussed in the appropriate sections of the text.

B. Quantitation of Lung Growth by Weight and Volume

Lung growth is often assessed by the measurements of lung weight and lung volume, usually relative to body weight since body size appears to influence the growth of the lung (1,6,75,77). Growth, with regards to lung weight, implies an increased size brought about by increased number and/or size of the cells on the one hand or by increased amount of the
intercellular connective tissue matrix on the other hand. In addition, the lung has the unique ability to increase in size without necessarily increasing in weight, this being brought about by increasing the air that the lung can contain per gram of tissue. This aspect of growth may be reflected in lung volume measurements. However, the growth in weight and in volume may show considerable disassociation because of altered distensibility of the lung and thus varying degrees of inflation (1,11).

The results of this study indicated that body weight, absolute lung weight and absolute lung volume were reduced in undernourishment and in experimental diabetes. However, in the experimental diabetic animals, the reduction in body weight was more extensive than that in lung weight and resulted in increased lung weight-to-body weight ratio (specific lung weight), whereas in the undernourished control groups, the lung weight-to-body weight ratio was similar to normal control values. This suggested that lung weight relative to body size was preserved in undernourishment but increased in the diabetic animals. The constant wet weight/dry weight ratios in all the experimental groups of animals indicated that the increase in specific lung weight of the diabetic animals was real and not due to altered moisture content. Moreover, it is not likely that the increase in specific lung weight is due to blood content since the lungs had undergone extensive blood vessel perfusion prior to the measurement of lung weight and thus were reasonably blood-free. It appears that experimental diabetes may also have an enhancing effect on specific weight of other organs since a similar
increase in tissue weight has been demonstrated in the kidney (134) and in the jejunum and ileum (135) of the experimental diabetic rats.

With regards to lung volume in the untreated diabetic animals, the reduction of body weight was also extensive compared to that of fixed lung volume and resulted in increased lung volume per unit body weight (specific lung volume). In contrast, lung volume per unit body weight in the undernourished control animals was similar to the normal control values. This suggested that lung volume appeared to be also preserved in undernourishment but increased in experimental diabetes. In this regard, it is apparent that specific lung weight and specific lung volume are increased in experimental diabetes, although the absolute values show some reduction when compared to rats with normal somatic growth. However, it is important to emphasize that fixed lung volumes may be complicated by the fact that they actually express fixative plus tissue volumes and thus are reflections of both the distensibility of the lung and the interaction between fixative and the connective tissue matrix of the lung tissues.

In order to determine the effect of changes in distensibility of the lung on the measured values of fixed lung volumes, it was necessary to determine the amount of fixative per gram of lung. The amount of fixative used to fill the lung air spaces per gram of tissue gives some, although not the best, estimate of the distensibility of the lung. If these were air-distended lungs, the measurements would be dealing with
excised lung mechanics. The data of fixative per gram of lung tissue (see Table IV) indicated that this parameter was increased in undernourishment but decreased in experimental diabetes. This suggested that the air spaces in the lungs of the undernourished control animals may be more distensible than those of the normal control animals, and agrees with the increased distensibility of air-filled lungs observed by Sahebjami et al (79,80) in food-deprived adult rats. The reduced fixative volume in the lungs of the untreated diabetic animals would thus indicate that the distensibility of the air spaces is reduced in experimental diabetes, and this may be the responsible factor in the reduced total lung capacity observed by Schuyler et al (97) in young men with juvenile-onset diabetes mellitus.

The finding of reduced lung distensibility but increased specific lung volume in the untreated diabetic animals suggested that the changes in specific lung volume in experimental diabetes may be due to increase in lung tissue. In contrast, the normal specific lung volume associated with increased distensibility of the lung in undernourishment has to be due to reduction in the amount of lung tissue. Interestingly, fixed lung volume per unit lung weight was decreased in experimental diabetes but increased in the undernourished control animals (see Table IV). Since volume per unit weight is the reciprocal of density, the data would seem to indicate that the density of lung tissue is indeed decreased in undernourishment but increased in experimental diabetes. Specifically stated, the increased specific lung volume in the untreated diabetic
animals appears to be a reflection of an increased amount of lung tissue.

The insulin-treated diabetic animals presented some interesting findings in lung weight and lung volume measurements. In agreement with the parameters of body size, insulin therapy adequately normalized the absolute and specific values of lung weight and fixed lung volume in the experimental diabetic animals (see Table IV). However, the fixed lung volume per unit of lung weight and the amount of fixative also relative to lung weight were midway between the untreated diabetic and the normal control values. This indicated that the increased amount of lung tissue and the reduced lung distensibility in experimental diabetes may not have been effectively controlled by the insulin treatment. Since lung weight and lung volume were normalized, it may be that some of the structural substrates of lung weight and lung distensibility were selectively restored while some others remained static and irreversible. Biochemical estimations of lung components would help clarify such possibility.

C. Cell Number, Cell Size and Connective Tissue Content of the Lungs

As indicated earlier, changes in lung weight may be a reflection of changes in number and size of the cells and/or in amounts of the intercellular connective tissue matrix. Cellular and connective tissue components of the alveolar wall make up the gas exchanging lung parenchyma, which represents approximately 90% of the total mass of the lung (100). The major airways and blood vessels comprise the remaining 10%. There are four major cellular components of the alveolar wall,
excluding the transient macrophages, known to have important functions in the normal lung. The alveolar surface in contact with air is lined by two highly specialized types of epithelial cells, designated alveolar epithelial types I and II cells. The alveolar epithelial type I cells, characterized by very thin cytoplasmic extensions which line approximately 97% of the alveolar surface area, are anatomically specialized to provide a minimum barrier to gas diffusion but have limited, if any, ability to proliferate and replicate. These cells represent 9.1% of total lung cells. The alveolar epithelial type II cells which represent 14.6% of total lung cells have two major functions that are critical in maintaining the integrity of the alveoli, namely the production of surfactant and the ability to readily proliferate and differentiate into type I cells when these are damaged by injury. These cells provide 2.9% of the alveolar surface area. Endothelial cells line the extensive intra-alveolar capillary network and make up 35.7% of the total lung cells. These cells are permeable to water and electrolytes but to some extent form a barrier to high molecular weight solutes. Moreover, a number of bioactive substances such as bradykinin, norepinephrine and serotonin are inactivated during passage through the lung by these endothelial cells. Mesenchymal cells, of which the fibroblasts and myofibroblasts are very prominent, are found within the interstitial tissue of the alveolar wall and represent 37.8% of the total lung cells. These cells are important in the synthesis of the major connective tissue proteins found in the alveolar wall.
In the present study, biochemical measurements of DNA content and of protein/DNA and RNA/DNA ratios in the lungs were used as recommended in other studies (1,13-15) as indices of cell number and cell size, respectively. The amount of DNA in every cell of the body is constant, and hence the DNA content of tissues adequately describe the number of cells in that particular tissue. In this regard, the data (see Table V) indicated that absolute cell number in the lungs of the untreated diabetic rats was reduced when compared to normal control values but was relatively normal when compared to the values of the weight-matched undernourished control animals. Moreover, when the absolute values of lung DNA were normalized for the change in body weight, it was apparent that undernourishment and experimental diabetes may not have altered specific lung DNA content (i.e. DNA/lung/body weight). It thus appeared that somatic growth and proliferation of lung cells may be related and that changes in cell number may not be responsible for the alteration in amount of lung tissue in undernourishment and in experimental diabetes. Since the amount of DNA per gram of lung tissue was increased in the undernourished control animals but reduced in the untreated diabetic groups, it would appear that alterations in cell size and/or in amount of connective tissue matrix may be more significant in producing the changes in amount of lung tissue.

With regards to protein/DNA ratios, the lung cells of the diabetic animals were similar to those of the normal control animals but appeared larger when compared to the undernourished control values. However, the
use of protein/DNA ratio as an index of cell size should be clearly defined since the amount of protein usually measured includes both cellular and extracellular proteins, particularly blood and connective tissue proteins. In this study, blood proteins may not have contributed to the protein values since the lungs were relatively depleted of blood by perfusion of the blood vessels. However, the contribution of the connective tissue proteins to the estimated protein values may complicate the interpretation of protein/DNA ratio as an index of cell size. Hence corrections were made for the connective tissue proteins in protein/DNA measurements. However, despite such correction it was still apparent that lung cell size in experimental diabetes is unaltered compared to normal controls but increased when compared to undernourished controls with similar somatic growth.

The RNA/DNA ratio, an index of cell size which is not complicated by extracellular factors, was decreased in the lungs of the undernourished control animals but appeared unchanged in those of the untreated diabetic group. However, the values of the untreated diabetic animals was increased compared to those of the undernourished controls. The finding of reduced biochemically-determined lung cell size in response to undernourishment are consistent with those reported by other investigators (81,138) in the lungs of starved adult rats. It is thus apparent that the reduced amount of lung tissue in undernourishment may be partly due to a reduction in the size of the cells. However, cell size did not appear to have any role in the increased amount of lung
tissue associated with experimental diabetes.

Morphologic and morphometric studies have indicated that cells of the alveolar wall were altered in experimental diabetes. Plopper and Morishige (105) described the dilatation of cytoplasmic organelles, such as the endoplasmic reticulum and the Golgi apparatus, in alveolar epithelial type II cells of the lungs of adult streptozotocin-diabetic rats, while Sugahara et al (107) demonstrated reduced number of the surfactant-storing lamellar bodies in the same cells in response to alloxan-induced diabetes in adult rats. Electron microscopic morphometry of the lungs of growing streptozotocin-diabetic rats indicated that the volume proportions of endothelial cells of the alveolar wall were increased (101). There was also a trend for the volume proportion of the mesenchymal cells to be larger than that in the control animals. There were no quantitative morphometric data of the lung cells in these studies of experimental diabetes. Morphometrically-determined volume proportion of the cells is a direct function of the product of cell number and cell size and an inverse function of the volume of the lung. Since the data in the present study seem to indicate that cell number and cell size are relatively spared in experimental diabetes, it appears that the morphometrically-determined increase in volume proportion of endothelial and mesenchymal cells in experimental diabetes may be due to the reduction in absolute volume of the lungs.
Since cell number and cell size do not seem to play any discernible role in the increase of lung tissue in experimental diabetes, the changes in connective tissue may be important. The data (see Table V) indicated that the amount of collagen and elastin in the lungs were increased in the untreated diabetic animals. When the amounts of these connective tissue proteins in the lungs of the untreated diabetic animals were normalized for the change in body size, the values indicated 145-147% of the normal control values, very close to the specific lung weight value of 131% compared to the normal control values. Thus the increase in specific lung weight in the experimental diabetic animals appears to be more closely associated with the increase in the connective tissue matrix. Moreover, the proportion of connective tissue to cellular elements in the lungs of the diabetic animals was markedly increased compared to the normal control values. The increase in collagen and elastin was similar, suggesting that these connective tissue proteins are equally important in increasing the amount of lung tissue in experimental diabetes.

In a previous study (101), increased volume proportions of basal lamina, collagen and elastin was demonstrated morphometrically in the alveolar walls of streptozotocin-diabetic rat lungs. Similarly, increased thickness of alveolar epithelium and capillary basal lamina have been indicated in the lungs of alloxan-diabetic rats (107) and those of human diabetics (90). Thus biochemically, the present study has confirmed the morphometric and morphologic increase in the amounts of
collagen and elastin in the lungs of the streptozotocin-diabetic rats whether compared to the normal control or to the undernourished control values; the increase was more pronounced when compared to the values of the undernourished control animals. Although the connective tissue proteins, particularly collagen, are heterogenous in anatomical location and type, the biochemical measurements of collagen and elastin in the present study may reflect the parenchymal tissue components since these comprise about 65% of the total lung connective tissue (100).

Collagen accounts for about 20% of the dry weight of the lung. Its heterogenous nature is particularly interesting. Type I collagen, composed of two alpha 1(I) chains and one alpha 2(I) chain, is the most abundant lung collagen. The tensile strength of this collagen type and its prominence in supporting tissues of the lung parenchyma strongly suggest that it is of major importance to the structural and mechanical properties of lung parenchyma. Type II collagen, composed of three identical alpha 1(II) chains, is confined to cartilagenous structures of the trachea and larger bronchi. Cartilage is not present in bronchi beyond the ninth division, and thus type II collagen is not detectable when lung devoid of these structures is studied. Type III collagen is composed of three alpha 1(III) chains and represents about 30% of the adult lung parenchymal collagen. This collagen type is abundant in pliable tissues such as blood vessels and gut, and since its proportion increases in fetal skin, in wound healing and in the developing lung, it has been suggested that type III collagen may be important in maintaining
the structural integrity of developing or remodelling lung tissues as well as contributing to their mechanical properties. Type IV collagen, a major component of alveolar and capillary basement membrane, is composed of two distinct chains, alpha 1(IV) and alpha 2(IV). In addition to the presumption that type IV collagen is necessary for the structural integrity of the alveolar-capillary basement membrane, it may also have nonstructural importance such as serving as a subsratum of epithelial cell attachment and migration (158).

Interestingly, Kern and Regnault (159) have demonstrated that the proportion of type III collagen relative to the type I was increased in the skin of the hereditary diabetic KK mice. Assuming that this diabetes-induced alteration is not restricted to skin alone, it is likely that the proportion of type III to type I in the lungs of the streptozotocin-diabetic rats may be altered as well. If this is what has actually happened, then most of the observed increase in the lung collagen of the untreated diabetic animals in the present study may reflect those of type III collagen and possibly of the type IV collagen, and would thus suggest enhanced developmental and/or remodelling processes in the lungs of the experimental diabetic animals.

In contrast, the absolute and body size-normalized content of lung collagen and elastin and also the proportion of connective tissue to cellular elements in the undernourished control animals were reduced.
Reduced content of lung collagen and of elastin in food-deprived adult rats have also been demonstrated by Sahebjami et al (84). These findings in food deprivation suggest that the nutritional abnormalities inherent in experimental diabetes may have affected the specific enhancing effects of diabetes on connective tissue deposition in the lung to some degree. Madia et al (83) have indicated the differential effects of food deprivation and experimental diabetes on lung acetic acid-soluble hydroxyproline (suggesting likely alteration in connective tissue formation) and lysyl oxidase activity, the enzyme responsible for the cross-linking and maturation of collagen and of elastin. Food deprivation reduced the content and activity of these biochemical factors while diabetes enhanced them. Thus it is apparent that experimental diabetes specifically increased the amount of the lung connective tissue proteins despite the stunted somatic growth, and hence the increase in specific lung weight. How this affects lung growth and structure is interesting and will be discussed later.

Again, the insulin-treated diabetic animals presented some interesting data (see Table V). The dose of insulin given to the diabetic animals increased the amount of DNA per lung from a depressed level to normal control values and increased further the amount of collagen per lung but did not change the diabetes-induced increase in the amount of elastin per lung. Hence, it was not surprising that the proportion of collagen to DNA was increased further while that of elastin to DNA was normalized in the lungs of the insulin-treated diabetic
animals. Moreover, this explains why the amount of DNA per unit lung weight was greater than the normal control values, while the amount of collagen and of elastin per unit lung weight was midway between the untreated diabetic animals and the normal control group. Insulin therapy also increased the amounts of total proteins, non-connective tissue proteins and RNA relative to DNA beyond the normal control values, indicating increased cell size. However, it also indicates that insulin therapy may have increased the amount of proteins in the lung tissue. A possible reason for these effects of insulin therapy is that this hormone stimulates DNA synthesis by lung cells (108) and has an anabolic effect on tissue proteins (86). However, the stimulation of growth hormone by hypoglycemia produced by excess insulin (86) may also be important, since growth hormone appears to cause hypertrophy of cellular and structural components of the lung (14).

Since insulin treatment of the diabetic animals did not seem to affect the increased content of collagen and of elastin per lung of these animals, it would appear that once these connective tissue proteins are formed in experimental diabetes, they remain static and irreversible and may not be controlled by insulin therapy. However, other components of the lung tissue such as cell number and also somatic growth were adequately restored. Hence the insulin therapy almost, but not completely, normalized the increased lung content of collagen and of elastin relative to body weight in the diabetic animals. Thus it appears that the insulin-treated diabetic animals attempt to match the
diabetes-induced increase in amount of connective tissue matrix by
increasing other factors such as cell number, cell size and somatic
growth.

D. Morphometric Data

1. Volume Proportion of Structural Components of Lung:

The morphometric data of lung structure (see Table VI) indicated
reduction in the volume proportion of alveolar air with a concomitant
increase in the proportion of alveolar wall in the lungs of the untreated
diabetic animals. The volume proportion of alveolar duct air was
unaltered. Cumulatively, these changes resulted in reduced proportion of
air spaces (alveolar air plus alveolar duct air) to alveolar wall in the
lung parenchymal structure of the untreated diabetic animals (3.69 ± 0.10
compared to 5.40 ± 0.15 for the normal control group). Since the volume
proportions of conducting airways and of nonparenchymal tissue were
unaltered, it suggests that the effects of experimental diabetes may be
restricted only to the lung parenchymal structures. Although
morphometrically determined volume proportion of lung structures is an
estimate since no correction has been made for the thickness of the
tissue sections, the change in the proportion of air spaces to alveolar
wall in the lungs of the diabetic animals seem to agree with the
estimated index of lung distensibility. Thus the thickened alveolar
walls and the reduced distensibility of the peripheral air spaces may
represent the most significant structural alterations in experimental
diabetes that are likely to affect lung function. Although about 20% of
the parenchymal tissue in normal mammalian lungs is comprised of
connective tissue (100), the increase in net amount of collagen and of
elastin and in the proportion of these connective tissue proteins to
cellular elements may represent the major contributing factor in the
increased thickness of the alveolar walls observed in human and
experimental diabetes.

In contrast, the volume proportions of all the lung structures in
the undernourished control animals were similar to those of the normal
controls. Surprisingly, the proportion of alveolar air plus alveolar
duct air to alveolar wall (5.31 ± 0.19) was not significantly different
from the values of the normal control animals and thus did not agree with
the increased index of lung distensibility observed in these animals.
Not correcting for the thickness of tissue sections in the estimation of
volume proportions may be responsible for this discrepancy, since this
will overestimate the volume proportion of alveolar walls and
underestimate that of the air spaces. Nonetheless, the findings in
undernourishment help establish that the diabetes-induced changes in
volume proportion of the parenchymal structures are unrelated to
nutrition and somatic growth.

However, despite the differences between the undernourished control
and untreated diabetic animals in volume proportions of the lung
parenchymal structures, the absolute volumes of alveolar air and of
alveolar duct air in the lungs were similar, although lower than normal control values (see Table VII). This was likely due to the disproportionate diminution in lung volume observed in response to undernourishment and experimental diabetes. Since the absolute lung volume is determined by the number and size of the air spaces, it is apparent that both parameters are affected differently by undernourishment and experimental diabetes. This will be discussed further under "Internal Structure of the Lung".

Interestingly, the specific volumes of alveolar air and of alveolar duct air were similar amongst the experimental groups of animals. This indicates that the changes in volume of the air spaces were commensurate with somatic growth. Burri and Weibel (6) have shown that the specific volume of alveoli plus alveolar ducts in normal developing rat lungs does not change from day 21 to day 44 of postnatal growth. Since the present study was conducted during a similar period of growth, the data further indicate that undernourishment and experimental diabetes do not seem to alter the normal development of specific volume of the air spaces. However, the specific volume of alveolar wall in the untreated diabetic animals was 147% of the normal control values. Since this parameter was unaltered by undernourishment, it is likely that the diabetes-associated increase in alveolar wall volume is diabetes-specific and unrelated to nutritional abnormality and stunted somatic growth. The increased alveolar wall volume approximated those of specific lung weight, volume and connective tissue content, indicating that the increased amount of
lung tissue in experimental diabetes may indeed be due to the increase in alveolar wall components, particularly connective tissue proteins.

The insulin-treated diabetic animals had values of volume proportions of alveolar air and alveolar duct air and of absolute volume of alveolar air that were midway between those of the untreated diabetic animals and the normal control group. However, the absolute volume of alveolar wall was further increased. This is not surprising, considering that the insulin therapy increased the cell number in the lungs of the diabetic animals to the levels observed in the normal control animals without reducing the diabetes-induced increase in connective tissue content. In fact, the amount of lung collagen was further increased in these insulin-treated diabetic animals. This inability of insulin treatment to control the increase in lung connective tissue and absolute volume of alveolar wall in experimental diabetes deserves attention since it is likely to alter the internal structure of the lung. This will be discussed further in the next section.

2. Internal Structure of the Lung:

In the untreated diabetic animals, the qualitative morphometric data (see Table VIII) indicated that the mean linear intercept (Lm), an estimate of the mean size of the peripheral air spaces at maximum inflation, was reduced. This alteration did not seem to be confined to alveoli or alveolar ducts, since the mean chord lengths of both structural units appeared to be equally affected. The change in size of
the peripheral air spaces agreed with the reduced index of lung
distensibility, thus indicating a likely cause and effect relationship
between both parameters. Although the absolute value of alveolar surface
area was also reduced, the values normalized for body weight and for lung
volume were increased, suggesting that the depressed absolute alveolar
surface area was likely a reflection of the reduced absolute lung volume
associated with stunted somatic growth in experimental diabetes.
Furthermore, the fact that alveolar surface area was increased relative
to lung volume indicated that the internal complexity of the lung has
been increased in the untreated diabetic animals. This could only have
occurred as a result of finer subdivisions of the internal structure of
the lung. There is only one way this could be possible; more alveoli are
formed (1,6,7). Hence it is not surprising that the number of alveoli
per unit lung volume in the untreated diabetic animals was 136% of the
normal control values, although the absolute number of alveoli was only
107% due to the smaller lung size. Thus it is apparent that experimental
diabetes produced more but smaller alveoli.

In contrast, the size of the peripheral air spaces in the
undernourished control animals was increased and this was in accordance
with the high distensibility of the air spaces. However, the absolute
surface area and that relative to body weight and to lung volume were all
reduced, indicating that the internal structure of the lungs was less
complex compared to normal controls. Hence the absolute number of
alveoli and the alveolar number per unit lung volume were 65% and 88% of
the
normal control values, respectively. Thus it is apparent that
undernourishment and experimental diabetes produced dissimilar
disturbances in the internal structure of the lung; undernourishment
depressed lung complexity and alveolar formation while experimental
diabetes enhanced these structural parameters.

Insulin deficiency in diabetes may be significant in the altered
internal structure of the lung, since insulin treatment of the diabetic
animals almost restored the changes in size of the air spaces and
alveolar surface area to normal control values. Although the number of
alveoli per unit lung volume in the insulin-treated diabetic animals was
midway between that of the untreated diabetic animals and the normal
control group, the absolute number of alveoli was not different from
those of the untreated diabetic animals. The basis for this inability of
insulin therapy to control the diabetes-induced increase in number of
alveoli per lung will become apparent in later discussion.

The finding that experimental diabetes altered the number of
alveoli and internal complexity of the lung is not completely new. A
previous study by Kida et al (101) indicated similar changes in the lungs
of streptozotocin-induced diabetic rats that only manifested mild
hyperglycemia and that had body weights similar to those of the control
animals. Since reduced body weight is a frequent and well-established
characteristic of experimental diabetic animals (86,110-115), this raised
the question of whether the effects observed in that study were indeed
diabetes-induced, particularly since that study failed to include a group of insulin-treated diabetic animals. Compared to the values of the undernourished control animals with similar body weights and nose-tail lengths, the diabetes-induced morphometric changes observed in the present study are more dramatic than those observed by Kida and associates. While they observed no change in absolute surface area in the lungs of the untreated diabetic rats with similar body weight as the controls, the present study indicated a 21% increase over that of the undernourished control animals. Although they found a 14% increase in surface-to-volume ratio, the present study observed a 48% increase. They indicated 23% and 29% increments in the absolute number of alveoli and the number of alveoli per unit lung volume, respectively, while the present investigation showed 66% and 54% increments, respectively. The present study also showed that a small but significant increase in absolute number of alveoli was apparent in lungs of the streptozotocin-diabetic rats that weighed 75% of the normal control body weights. Even the insulin-treated diabetic animals had more alveoli per lung than the normal control animals. Thus the present study established more clearly that experimental diabetes increased the number of alveoli and hence the more complex internal structure of the lung.

With regards to the effect of undernourishment on the internal structure of the lung, Sahebjami and Vassallo (80,160) have also indicated that the size of the peripheral air spaces was increased while the internal surface area was decreased in adult rats that only received
one-fifth of their usual daily food consumption for ten days. Their data indicated that the internal surface area per unit lung volume in these starved rats was about 65% of the values in the normal fed group. D'Amours et al (85) studied the lungs of adult rats similarly deprived of food for 3 days and observed no statistically significant change in lung volume and alveolar surface area. They did not estimate alveolar surface area-to-volume ratio. However, when their data are calculated, there was a tendency towards decreased alveolar surface area per unit lung volume in these food-deprived rats, although this may not reach a significant level for accurate interpretation. These investigators did not choose to use their data to suggest that the internal complexity and thus the number of alveoli may be decreased in their food-deprived rats. The data of the present study indicated that this is what appears to occur in the undernourished control rats. Thus the estimation of absolute number of alveoli and the alveolar number per unit lung volume in the present study represents the first precise indication of reduced number of alveoli in the lungs of undernourished rats. Such finding has some pathophysiological significance, since the changes in lungs of food-deprived rats have been likened to those which occur in emphysema (79,80). It is very likely that defective alveolar formation rather than loss of alveoli may be the major contributing factor in the decreased internal surface area observed in food-deprived animals.

Alveolar formation is predominantly a postnatal event. Hence postnatal lung growth and development is characterized by increases in
alveolar number and maturation of their structures (1-7,10,11). The unresolved question is when new alveoli cease to form in the developing postnatal lung. According to Burri and associates (161), rapid tissue proliferation in rat lungs occurs from day 4 to 13 of postnatal life. During this period of growth, the absolute number of alveoli increased 10-fold; alveolar surface area increased to the 1.6 power of lung volume, a rate compatible with alveolar formation. After the second week, the alveolar surface area only increased to the 0.71 power of lung volume, very close to the 0.67 power that would come by simple enlargement of the existing alveoli. The number of alveoli only increased 1.3-fold from day 13 to 21; alveolar number was not determined thereafter. However, Holmes and Thurlbeck (7) have shown that alveolar surface area increased directly with lung volume from 4 to 14 weeks of postnatal life in rats. Their data indicated that the absolute number of alveoli increased about 2-fold from 4 to 10 weeks with some accompanying increase in alveolar dimension.

Burri and associates were primarily concerned with lung growth in rats from birth up to day 21 of postnatal life, but supplemented their data with those for 44-day and 131-day old rats from another study; while Holmes and Thurlbeck only studied postnatal lung growth after the fourth week of life. However, some apparent differences between both studies may be responsible for the discrepancy in their estimates of alveolar surface area-to-volume ratio. The 44-day old rats of Burri and associates were about 50% smaller than the 42-day old rats that were part
of the age groups studied by Holmes and Thurlbeck, and lung volumes were almost twice as small. The light microscopic data of alveolar surface area in the 42-day old rats of Holmes and Thurlbeck was larger compared to that determined by electron microscopy in the 44-day old rats of Burri and associates. These differences in animal and lung sizes in both studies might have been due to the fact that different strains of rats were studied and may explain why alveolar surface area was 0.71 power of lung volume in the adult rats of Burri and associates but directly proportional to lung volume in those of Holmes and Thurlbeck. However, based on alveolar counts performed in both studies, it appears that alveolar multiplication continues for a considerable period of postnatal life in adult rats.

The present study may shed some more light into the question of when does alveolar multiplication cease in postnatal rat lungs. If alveolar formation had ceased by the third week of postnatal life, then the effects of undernourishment and experimental diabetes on growth of the lung from 3 to 7 weeks of age should not involve alterations in the absolute number of alveoli, unless there is actual destruction of alveoli; only alveolar size should be affected. This is contrary to the findings in the present study. The increase in number of alveoli per lung of the experimental diabetic animals was accompanied by reduction in the size of the alveoli, whereas the reduced alveolar number in the lungs of the undernourished control animals was associated with increased alveolar size. These alterations suggest that the pattern of lung growth
from 3 to 7 weeks has to be that of alveolar multiplication and enlargement for both to be affected, and thus is in agreement with the findings by Holmes and Thurlbeck (7) in normal posnatal lung growth of the rats. However, it is apparent that undernourishment and experimental diabetes have differential effects on alveolar proliferation. What is the basis for these differences?

A major hypothesis of alveolar formation is that the septal network of elastic fibers and collagen provide some kind of anchorage points through which the lung parenchyma protrudes to form the alveolar outpouchings; this is often termed the "fishnet theory" (1,5,6). This hypothesis is based on the characteristic predominance of elastic fibers, in particular, at the tips of newly developing secondary crests and the mouths of mature alveoli. In support of this role of connective tissue in alveolar proliferation, Kida and Thurlbeck (30,31) have demonstrated that the connective tissue cross-link inhibitor, B-aminopropionitrile, interferes with alveolar formation. In the present study, the data would seem to suggest that the increased amounts of elastin and collagen in the untreated diabetic animals resulted in the increased number of alveoli; in contrast, the decreased amount of these connective tissue proteins in undernourishment produced few alveoli. The finding that insulin treatment of the diabetic animals did not alter the diabetes-induced increase in elastin and collagen explains why the absolute number of alveoli in the lungs of these animals was similar to those of the untreated diabetic group. Since elastin and collagen were always
similarly altered in this study, it is likely that both connective tissue proteins were equally important in the altered number of alveoli observed in undernourishment and experimental diabetes.

According to Kauffman et al (162), alveoli are formed by cellular proliferation in the secondary crests. Conceivably, if cellular proliferation is decreased, then the crests would not develop properly and hence a diminution in number of alveoli would result. In the present study, the absolute content of lung DNA was similarly reduced in both experimental diabetes and undernourishment (see Table V), yet the absolute number of alveoli was increased in the former although decreased in the latter (see Table VIII). Insulin treatment of the diabetic animals normalized the DNA content but did not alter the diabetes-induced increase in number of alveoli. These changes in experimental diabetes would seem to favour the role of connective tissue rather than of cellular proliferation in the process of alveolar formation, and hence provide support to the "fishnet theory". However, in the lungs of the undernourished control animals, cell number and amount of connective tissue were equally reduced as the number of alveoli, indicating that the importance of connective tissue deposition in alveolar formation is not as explicit in undernourishment as in experimental diabetes.

The observed changes in the internal complexity of the lung in experimental diabetes are particularly interesting with regards to lung function. Sugahara et al (96) have indicated that lung carbon monoxide
diffusing capacity is often lower in diabetic patients when compared to that in a corresponding age group. The rate at which gas moves across the alveolar-capillary membrane is a function of alveolar surface area and thickness of the alveolar-capillary membrane (158). In the present study, the data indicated that alveolar surface area relative to body size and to lung volume was increased in the experimental diabetic animals. Such findings suggest that the altered gas exchange property in diabetics may not have been due to changes in alveolar surface area, leaving any alteration in the alveolar-capillary membrane as a likely culprit. The alveolar-capillary membrane, physiologically described as the blood-air barrier, is comprised of alveolar epithelial cells, epithelial basement membrane, interstitial connective tissue materials, capillary basement membrane and capillary endothelial cells. These structures could pose a serious diffusion barrier to gas exchange if they were increased. It is thus likely that, despite the increased alveolar surface area in the lungs of the diabetic animals, the increased volume and thickness of the alveolar wall and amount of connective tissue observed in the present study may be responsible for the abnormal gas exchange function in diabetics.

Another important finding that emanated from the present study is the relationship between alveolar surface area and pulmonary surfactant in experimental diabetes. Normal mechanical function of the lung requires that adequate amounts of surfactant be present over the alveolar surface. Recent studies (103,104,136) have demonstrated that the amount
of lung phospholipids and surfactant materials are reduced in experimental diabetes, and suggested that altered mechanical functions may result from these changes. These studies made no attempt to relate these changes to the surface area available in the lung. Surface balance studies by Kida et al (101) found no significant differences in surface tension and stability index of lung surfactant in control and experimental diabetic rats. The data of present study (see Table IX) demonstrated that the total lung phospholipid and amount of disaturated phosphatidylcholine, a marker for pulmonary surfactant (18-21), were decreased in experimental diabetes. These findings are in agreement with those of other studies (103,104,136). However, when the changes in the amount of surfactant material recovered from the airways were expressed relative to the alveolar surface area of the lung, no differences were observed between the control and diabetic animals. This indicated that the changes in surfactant levels were compatible with those of alveolar surface area of the lungs, thus suggesting that the altered pulmonary surfactant levels in experimental diabetes is unlikely to produce a significant change in the mechanical properties of the lung.

The effect of diabetes on lung mechanics in humans remains controversial. Schuyler et al (97) demonstrated reduced elastic recoil in young men with juvenile-onset diabetes mellitus, contrary to the findings by Schernthaner et al (98). Of the factors that contribute to the mechanical properties of the lung, the connective tissues of the lung, primarily elastin and collagen, have long been known to be
important determinants. However, the surface tension of the liquid film of surfactant lining the alveoli also exerts an effect. Saline-filled lung (with surface tension forces thereby abolished) is twice as compliant as air-filled lung, indicating that surface tension contributes a large part of the static recoil force of the lung (158). In the study of experimental diabetic rats, Kida et al (101) were unable to show any changes in air-filled pressure-volume characteristics of the lung. Since no changes were present in their surface activity studies, these investigators suggested that the connective tissue properties were also normal in experimental diabetes. The present investigation did not attempt mechanical studies and thus is unable to clarify the effects of diabetes on lung mechanical functions. However, the fact remains that the amount of connective tissue was altered and definitely involved in the altered internal complexity of the lung in experimental diabetes.

E. Dynamic Aspects of Connective Tissue Metabolism

The changes in amount of connective tissue in the lungs of the experimental diabetic animals and the undernourished control group could be a function of synthesis, degradation, or a combination of both processes. The present study was particularly concerned with likely changes in connective tissue synthesis based on the extent of labelled proline incorporation into hydroxyproline. Throughout the study, it was assumed that hydroxyproline in the lungs of the experimental animals was unique to the connective tissue proteins, and thus hydroxyproline activity was regarded as a good index of connective tissue synthesis.
During the course of this investigation, it was apparent that a valid comparison of in vivo connective tissue synthesis between the experimental group of animals could not be made without taking into account the differences in the specific activity of the injected radiolabelled proline in the precursor pools. The lower serum proline specific activities after the injection of the radiolabel in the diabetic animals compared to that of the animals in the two control groups (see Figure 10) support the relevance of such consideration. Failure to do so would result in underestimation of connective tissue synthesis in the diabetic animals. Since the specific activity of a product at any time involve the integral of the specific activity of the precursor up to that time, the necessary correction factor for proline and/or hydroxyproline activities in the lung tissue of the control and diabetic animals could be obtained from the geometrical integration of the specific activity of serum proline in the animals, which is represented by the area under the serum proline specific activity curve. Other investigators (132,133) have used a similar principle and derived correction factor in studying connective tissue metabolism in the kidney of experimental diabetic animals.

The lower specific activity of serum proline in experimental diabetes may be the result of a simple dilution of the injected tracer dose by a larger proline pool arising from increased peripheral output of the unlabelled amino acid to meet the demands of gluconeogenesis. However, the maintenance of normal serum proline concentration in all the
experimental groups of animals in the present study would seem to indicate that uptake of proline may have been increased in the diabetic animals compared to the normal and undernourished control groups. Since the activity of the free proline pool in the lung tissue of the experimental group of animals was similarly altered (see Table X), uptake of proline may not be the cause of the alteration in the precursor pools in experimental diabetes. Whatever the reason may be, it was necessary that these differences be considered.

After application of the relevant correction factor for each experimental group of animals, a series of data for connective tissue synthesis were obtained. The first of these data (see Table XI) indicated that the total lung activity after 24 hours of proline incorporation in the untreated diabetic animals was approximately 85% of the normal control values, while that in the undernourished control animals was 57% of the normal control. Since proline incorporation is a measure of protein synthesis, the data suggested that protein synthesis was reduced in the lungs of the untreated diabetic and undernourished control animals, although more so in the latter group. Rannels et al (82) had demonstrated that protein synthesis, estimated in lungs perfused in situ with \[^{14}\text{C}]\)phenylalanine, was unchanged in adult rats that had been diabetic for 3-5 days; there was a 22% reduction in starvation. However, Morishige et al (102) showed that protein synthesis was reduced in growing streptozotocin-diabetic rats that were only diabetic for a similar period of time; a finding that is in agreement with that of the present study.
However, the particular interest of the present study was in the changes in connective tissue synthesis. The data (see Table XI) indicated that the total lung activity of $^{14}$C-hydroxyproline was reduced in the untreated diabetic animals, more reduced in the undernourished control group but increased in the insulin-treated diabetic animals. Because interpretation of such data depended on equating $^{14}$C-proline incorporation into $^{14}$C-hydroxyproline production as a measure of connective tissue synthesis, it was necessary to ascertain that the results did not represent alterations of proline hydroxylation rather than connective tissue synthesis. The data indicated that 5% to 6% of the total incorporation was present in hydroxyproline in the lungs of all the experimental groups of animals, suggesting that hydroxylation of proline was not affected by experimental diabetes and undernourishment. Thus the changes in activity of $^{14}$C-hydroxyproline are acceptable as representative of connective tissue synthesis. In this regard, it was apparent that connective tissue synthesis may be reduced in the untreated diabetic and the undernourished control animals, although more so in the latter group; while insulin treatment of the diabetic animals may increase the synthesis of this important lung component.

The data presented so far represented that of lung fractions soluble in phosphate-buffered saline. Although newly synthesized connective tissue proteins are relatively immature and thus very soluble in neutral salt medium such as phosphate-buffered saline, inherent
conditions may readily convert these proteins into the mature polymeric forms which are insoluble in neutral salt as a result of their increased molecular cross-linkages. The data of the present study (see Table XII) indicated that the changes in total activity of the soluble connective tissue \(^{14}\)C]hydroxyproline in the lungs of all the experimental groups of animals after 6 hours of proline label were similar to those observed after 24 hours. These changes were indicated even when activity was expressed relative to DNA. Similar alterations of connective tissue activities after 6 and 24 hours of proline label were also apparent in the insoluble fractions of the lungs, except in the untreated diabetic animals where the activity of the insoluble \(^{14}\)C]hydroxyproline per unit of DNA was not different from the normal control values. These findings confirmed that the observed changes in \(^{14}\)C]hydroxyproline activity were apparent in both the soluble and insoluble fractions of the lung, and thus established that connective tissue synthesis may indeed be greatly reduced in undernourishment, minimally depressed in experimental diabetes and this could be rectified by insulin treatment.

However, the present study further indicated (see Table XII) that the proportion of total \(^{14}\)C]hydroxyproline in the insoluble fraction of the lung was increased in the diabetic rats but decreased in the undernourished control animals. Conversion of soluble connective tissue proteins to the insoluble polymeric forms is representative of the extent of the molecular cross-linkages, and hence its estimation is often used as an index of connective tissue maturation (163,164). In this regard,
it became apparent that connective tissue cross-linkages and thus maturation was increased in the lungs of the untreated diabetic animals but decreased in those of the undernourished control group. This is particularly noteworthy since Madia et al (83) had also observed that the lung activity of lysyl oxidase, the enzyme responsible for the cross-linking and maturation of the connective tissue proteins, was increased in experimental diabetes but decreased in starvation.

In order to differentiate between collagen and elastin synthesis, the activity of $[^{14}\text{C}]$hydroxyproline solubilized after cyanogen bromide treatment of the insoluble lung fractions was taken to represent specifically collagen synthesis while the total activity ($[^{14}\text{C}]$proline plus $[^{14}\text{C}]$hydroxyproline) in the cyanogen bromide-insoluble material represented elastin synthesis. This method of analysis was based on the findings of Laurent et al (124) that indicated that cyanogen bromide completely solubilized lung collagen by breakage of peptide bonds at methionine positions, leaving an insoluble elastin residue; elastin does not contain methionine. In this study, the data (see Table XIII) indicated that collagen and elastin syntheses were similarly reduced in the untreated diabetic animals, although the activities were again greater than those of the undernourished control animals. This was an indication that the changes in connective tissue synthesis were not confined to one particular protein component, but equally distributed between collagen and elastin.
Independent studies have indicated that connective tissues respond differentially to diabetes. Glomerular basement membrane collagen of human and experimental diabetics showed increased amount and synthesis but decreased degradation (132,133,139). In skin and aorta, experimental diabetes resulted in decreased synthesis of collagen, with accompanying decreased amount in the skin but no effect on the aortic content (140). Thus the response of the rat lung connective tissue proteins in experimental diabetes appear to be similar to that of kidney collagen with regards to content, but similar to the skin connective tissue protein with regards to synthesis.

Studies of the kidney in experimental diabetes by Brownlee and Spiro (132) and Cohen et al (133) have indicated that the degradation of the glomerular basement membrane collagen was slower compared to those in control animals. Chang and associates (89) indicated that B-aminopropionitrile, a potent inhibitor of connective tissue cross-link formation, suppressed the diabetes-induced increase in maturation and stability of collagen in skin and possibly other organs. Since the present study indicated that there was an apparent increase in maturation of connective tissue in the lungs of the untreated diabetic animals, it is very likely that the increased content of the lung connective tissue proteins in experimental diabetes was probably a reflection of reduced degradation resulting from unusual highly stable cross-links in the molecules. Alternatively, it is possible that enhanced synthesis of the lung connective tissue proteins may have occurred earlier on and that the
mechanism was already switched off at the time the in vivo metabolic measurements were performed. If this is the case, then it would seem that experimental diabetes has but a transient effect on lung connective tissue synthesis.

In contrast, the reduced amount of lung connective tissue in the undernourished control animals may be due mostly to reduced synthesis. Moreover, only a smaller proportion of the newly synthesized connective tissue was present as the insoluble mature form, suggesting that there was depressed molecular cross-link formation, less stability and maturation of the connective tissue in the lungs of these animals. Such structural abnormality is very likely to render the connective tissue proteins to increased degradation. Thus the reduced amount of lung connective tissue proteins in undernourishment may also be due to enhanced degradation. Studies in other tissues (163,165) have similarly demonstrated reduced synthesis and maturation of connective tissue in starvation and protein malnutrition.

The dependence of connective tissue formation and deposition on growth rate of the animal has been demonstrated in various tissues, including the lung (37,133,165). Thus the dissimilar synthetic activities of lung connective tissue proteins in the untreated diabetic and the undernourished control animals is particularly noteworthy since these animals had similar somatic growth values. When the metabolic activities in the untreated diabetic animals are compared to those of the
undernourished control group, the data (see Tables XII and XIII) show approximately 2-fold increase in synthesis and even in maturation of the connective tissue proteins. Hence it is also likely that experimental diabetes may enhance lung connective tissue synthesis and deposition, but the inherent abnormal growth rate of the experimental diabetic animals may have obscured these effects, particularly that of synthesis.

The diabetes-induced increase in amount of the connective tissue proteins was not rectified by insulin therapy; in fact, the content of collagen was further increased (see Table V). The total synthetic activities of these structural components in the diabetic animals were increased in response to insulin treatment. However, the activity per unit lung DNA was only increased in the collagen component, with no apparent effect on elastin (see Tables XII and XIII). Maturation of the newly synthesized connective tissue to the less degradable form was apparently controlled. Thus it is apparent that insulin treatment of the diabetic animals increased lung collagen via enhanced synthesis. It is possible that the anabolic effect of insulin on tissue proteins (86) is only feasible with collagen and not with elastin. Alternatively, the stimulation of growth hormone by hypoglycemia produced by excess insulin (86) may also be important, since growth hormone appear to affect collagen formation and deposition (14). There are no available information in the literature regarding the effect of growth hormone on elastin metabolism.
F. In vivo Lung DNA and Connective Tissue Synthesis: Possible Association with Changes in the Cyclic AMP Modulatory System

The data of present study (see Tables V and XIII) indicated that DNA content and activity (indices of cell number and cellular proliferation, respectively) were similarly reduced in the lungs of untreated diabetic and undernourished control animals. However, in the untreated diabetic animals, collagen and elastin activities per unit DNA (representing the production of the connective tissue proteins per cell) were not altered, although total activities were reduced. These findings suggested that experimental diabetes only depressed total connective tissue production in the lung as a result of the suppressive effect on cell number. In contrast, collagen and elastin activities per unit DNA were reduced in the lungs of the undernourished control animals and hence the marked reduction in the total synthetic activities of the lung connective tissue proteins in these animals. Apparently, experimental diabetes and undernourishment may have a common effect on cell number but are definitely different with regards to their effects on the cellular capacity for connective tissue production. The biochemical basis for these differential effects needed to be sought and understood.

It has been shown that increases in lung fibroblast cyclic AMP levels induced by exogenous drugs, possibly via adenylate cyclase stimulation, resulted in a selective decrease in collagen production and cellular proliferation (43,44,141). Moreover, previous studies (23,50,51,54,58,60,110-112) have identified two factors in rat lung that
regulated cyclic AMP levels during normal lung growth, and the activities of these modulators were altered in response to experimental diabetes. One of these factors is the ubiquitous heat-stable 17000 dalton protein referred to as calmodulin. This Ca$^{2+}$-dependent regulatory protein activates adenylate cyclase (the cyclic AMP synthesizing enzyme) as well as cyclic AMP-phoshodiesterase (the cyclic nucleotide degrading enzyme). The other modulator which is composed of 14500 and 65000 dalton heat-labile proteins has been referred to as the adenylate cyclase cytosolic modulator, since it is located in the cytosol and specifically enhances lung particulate adenylate cyclase activity. Based on these findings, a major objective of the present study was to determine if the lung changes in cellular proliferation and connective tissue production in experimental diabetes and undernourishment (Table XIII) were closely associated with changes in levels of cyclic AMP and/or activities of the endogenous modulators of cyclic AMP formation and degradation (Table XIV).

In the lungs of the untreated diabetic animals, specific activity of DNA (dpm/mg DNA) was reduced by 20%, closely associated with a 21% increase in the cellular activities (per mg DNA) of the adenylate cyclase cytosolic modulator and a 23% reduction in that of calmodulin; the cellular content of cyclic AMP was increased 41% while the specific activities of collagen and elastin were not altered. The similar magnitude of change in specific activities DNA and in cellular activities of the adenylate cyclase cytosolic modulator and calmodulin suggested that cellular proliferation in the lungs of experimental diabetic animals
may be functionally associated with the activities of the cyclic AMP modulatory factors rather than with the cellular cyclic AMP levels. However, the 43% reduction in total activity of DNA was only close to the 39% reduction in total activity of calmodulin; the total activity of the adenylate cyclase cytosolic modulator was only increased by 4% and the total content of cyclic AMP was increased 10%. The total activities of collagen and elastin were depressed by 20% and 24%, respectively. Since only the magnitude of change in total activities of DNA and calmodulin were close, it would appear that the reduced activity of calmodulin may have greater modulatory relevance than the increased activity of the adenylate cyclase cytosolic modulator in producing suppressed cellular proliferation in the lungs of experimental diabetic animals. Neither of the endogenous modulators seem to be relevant in mediating the production of connective tissue proteins.

Brownlee (152) put forward the hypothesis that the reduced degradation of connective tissue materials in diabetes may be due to the decreased activity of polymorphonuclear neutral proteases caused by increased plasma \( \alpha_2 \)-macroglobulin. This hypothesis resulted from the observation of increased concentration of \( \alpha_2 \)-macroglobulin in the blood of diabetic patients and the inhibitory effect of this blood protein on the activity of purified neutral proteases. The stimulation of \( \text{Ca}^{2+} \)-dependent neutral proteases by calmodulin-enriched samples of bovine brain has been demonstrated (153). Morley et al (154) recently observed that diabetic patients have significantly lower
polymorphonuclear cell calmodulin activities than age-matched controls. Calmodulin has been implicated in normal neutrophil functions including chemotaxis (155) and phagocytosis (156). Abnormalities of chemotaxis, phagocytosis and bacterial killing have all been detected in leukocytes obtained from diabetic patients (157). It is thus likely that the reduced calmodulin activity in the lungs of the experimental diabetic animals may contribute to the increased lung content of collagen and elastin observed in this study by reducing their degradation, despite the unchanged synthetic activities of these connective tissue proteins.

However, the changes in the activities of these endogenous modulators in experimental diabetes may not be primarily responsible for the altered cellular proliferation and connective tissue deposition observed in vivo in the lungs. Reduced insulin levels in diabetes may come to mind as the primary defect. Insulin is known to have stimulating effects on synthesis of DNA (108) and of collagen (148) by fibroblasts in culture. Murphy and Lazarus (149) have demonstrated that serum from untreated streptozotocin-diabetic rats was significantly less potent than that from normal rats in stimulating DNA synthesis by lung fibroblasts in culture. Even serum from insulin-treated diabetic rats was less potent than normal rat serum. The impaired stimulation of the DNA synthetic activities did not appear to be due to small molecules such as ketones, since dialyzed serum from the untreated diabetic rats was less potent than dialyzed normal serum. The addition of normal rat serum and somatomedin-depleted (hypophysectomized) rat serum, but not insulin
alone, to diabetic rat serum resulted in the same degree of stimulation of DNA synthesis as that seen with normal rat serum. There was no evidence of an inhibitor of fibroblast DNA synthesis in the untreated diabetic rat serum. On the basis of these findings, these investigators suggested that the defect in cellular proliferation in diabetes mellitus may be due to depletion of circulating growth factors other than insulin and somatomedins.

Maquart et al (150) indicated that fibroblasts submitted to an increased glucose concentration of the medium comparable to the in vivo hyperglycemia in diabetes, react by a decrease in the synthesis of DNA but enhancement of collagen production. The increase in collagen synthesis was not specific since total protein synthesis was similarly enhanced. Duhaault et al (151) demonstrated a similar reaction for endothelial cells cultured in high glucose or sorbitol concentrations. The fact that different circulating factors may be primarily responsible for the altered DNA synthesis and collagen deposition in diabetes suggest that a common cellular mediator may be present. On the basis of findings in this study, it is very likely that calmodulin and the adenylate cyclase cytosolic modulator may represent the intracellular mediators of the possible circulating factors.

The lungs of the insulin-treated diabetic animals showed (Tables XIII and XIV) showed a much closer association between the synthetic activities and those of the endogenous modulators. Compared to normal
control values, the specific activity of DNA was increased 10%, very close to the 18% increase in activity of collagen per unit DNA, the 14% increase in cellular activity of calmodulin and the 13% decrease in cellular activity of the adenylate cyclase cytosolic modulator. The activity of elastin per unit DNA and the cellular content of cyclic AMP were not significantly different from the values of the normal control animals. However, the total activities of DNA, collagen and elastin increased by 13%, 19% and 15%, respectively; these changes were very close in magnitude to the 20% increase in total activity of calmodulin and the 10% decrease in that of the adenylate cyclase cytosolic modulator. These findings in the insulin-treated diabetic animals give credibility to the assumption that the effects of circulating factors such as insulin on lung DNA synthesis and connective tissue deposition may be mediated by endogenous calmodulin and/or the adenylate cyclase cytosolic modulator.

In the lungs of the undernourished control animals, the association between the cellular synthetic activities and those of the endogenous modulators do not seem to be clearly defined. Compared to normal control values, the specific activity of lung DNA was reduced 20% and the activities of collagen and elastin per unit DNA were reduced by 53% and 49%, respectively. Concurrently, the cellular activity of calmodulin decreased 36%, that of the adenylate cyclase cytosolic modulator increased 88% and the cellular content of cyclic AMP increased 129%. Only the change in calmodulin activity and cellular proliferation seemed
likely to have any close association. However, when total lung activities are considered with those of the endogenous modulators, some relationship seemed apparent. For example, the total activity of DNA was reduced by 43% and that of elastin by 49%, very close to the 48% decrease in total activity of calmodulin and the 50% increase in that of the adenylate cyclase cytosolic modulator. The 63% decrease in total activity of collagen and the 82% increase in total content of cyclic AMP may be functionally associated but based on the magnitude of change such association was not clearly indicated.

Although the present study has used the magnitude of changes in the lung synthetic activities and in those of the endogenous modulators in the experimental diabetic and undernourished control animals to suggest some association between the activities of calmodulin and the adenylate cyclase cytosolic modulator on the one hand and in vivo cellular proliferation and connective tissue production on the other hand, a cause and effect relationship was hard to establish. Moreover, it is very likely that extra-lung factors which do not have any relationship with the endogenous modulatory factors may have influenced the estimated synthetic activities. Such possibility was apparent in the estimation of connective tissue production where the changes in circulating activity of the precursor proline could have affected the in vivo synthetic activities, although corrections were made for the likely effects (discussed earlier). An in vitro approach was therefore employed in defining a cause and effect relationship between the lung synthetic activities and those of the endogenous modulators.
G. In vitro Effects of Soluble Lung Factors on Synthesis of Lung DNA and Connective Tissue Proteins

The data of the present study (see Figures 11 to 14) indicated that crude soluble lung extracts derived from the experimental diabetic and undernourished control animals suppressed DNA synthesis and connective tissue production by normal lung mince cultures in a concentration-dependent manner. The crude extracts derived from the lungs of undernourished control and untreated diabetic animals were similar in their suppressive activities on DNA synthesis but were more potent than those of the normal control and insulin-treated diabetic animals. However, the extracts of the undernourished control animals were more potent than those of the diabetic and normal control animals with regards to suppressive effects on both collagen and elastin production. In fact, the suppressive potency of the extracts on connective tissue production increased in the order: insulin-treated diabetic, normal control, untreated diabetic, undernourished control. These findings agreed with those observed in the in vivo lung synthetic studies of the experimental animals, and suggested that the factors present in the lung extracts probably mediated the in vivo changes in cellular proliferation and connective tissue production in the rat lungs in response to undernourishment and experimental diabetes. It is interesting that although the crude soluble lung extracts of undernourished control and untreated diabetic animals had similar suppressive activities on DNA synthesis, they differed in their inhibitory activities on connective tissue production, and this was an
indication that the regulatory factors may be heterogenous or differentially affected by undernourishment and experimental diabetes.

Initial characterization of the suppressive activities of the crude soluble lung extracts of all the experimental groups of animals indicated that the endogenous factors were sensitive to trypsinization and thus proteinaceous, were partly sensitive to 90\(^\circ\)C heat-treatment for 5 min and did not bind to DEAE-cellulose at 0.25 M NaCl at neutral pH (see Figures 15 and 16). Moreover, the suppressive activities were associated, in a concentration-dependent manner, with increased level of cyclic AMP in the lung minces (see Table XV). The addition of lung extracts from the undernourished control, untreated diabetic, normal control and insulin-treated diabetic animals to the standard lung minces produced 199%, 173%, 134% and 113% of the basal cyclic AMP values, respectively. This order of changes was similar to that observed in lung cyclic AMP contents in the in vivo studies. These findings became intriguing for several reasons. Firstly, it has been shown that the adenylate cyclase cytosolic modulator is heat-labile whereas calmodulin is heat-stable and elutes from DEAE-cellulose column at 0.2 to 0.3 M NaCl at neutral pH (23,54,153). Secondly, both endogenous factors enhance cyclic AMP formation and their effects are additive (58). Thirdly, increases in lung fibroblast cyclic AMP levels have been shown to result in a selective decrease in cellular proliferation and collagen production (43,44,141). Fourthly, Clark et al (43,142) have recently demonstrated in lung tissue the presence of a nondialyzable, heat-stable, 15000-20000
dalton protein factor which increased intracellular cyclic AMP levels of lung fibroblasts and suppressed their proliferation and collagen production.

Further characterization of the activities of the crude soluble lung extracts of all the experimental animals in the present study showed that essentially all of their stimulatory effects on cyclic AMP production and their suppressive effects on cellular proliferation and connective tissue production were accounted for in calmodulin-enriched and adenylate cyclase cytosolic modulator-enriched fractions of the lung extracts obtained by Sephadex G-75 column chromatography (see Figures 17 and Table XVI). The adenylate cyclase cytosolic modulator-enriched fraction (fraction II) of the undernourished control animals showed the greatest protein content and produced the largest suppressive effect on thymidine incorporation and hydroxyproline production. The fraction of the untreated diabetic animals was next in protein content and suppressive potency, followed by that of the normal control and the insulin-treated diabetic animals. However, with regards to the calmodulin-enriched fraction (fraction III), the untreated diabetic animals had more protein and suppressive potency on thymidine incorporation but least on hydroxyproline production. The fractions of the undernourished control, normal control and insulin-treated diabetic animals were similar in their suppressive effects on thymidine incorporation and hydroxyproline production. These findings seemed to indicate that the adenylate cyclase cytosolic modulator was more
prominent in suppressing cellular proliferation and connective tissue production in the lungs of the undernourished control animals, whereas calmodulin was more involved in reducing cellular proliferation in the untreated diabetic animals with least effect on connective tissue production. It is particularly interesting that a similar association was observed earlier in the in vivo studies, thus establishing that the endogenous lung modulators may have regulatory functions in cellular proliferation and connective tissue production in vivo.

Since the suppressive activities of the adenylate cyclase cytosolic modulator-enriched fractions were abolished by heat but not by trifluoperazine, a calmodulin inhibitor (see Figure 18), it is believed that the activities measured were specifically due to adenylate cyclase stimulation by the cytosolic modulator. However, the suppressive activities of the calmodulin-enriched fractions on thymidine incorporation appeared heterogeneous since trifluoperazine treatment which should abolish calmodulin activities rather increased further the suppressive effects of these fractions. On further characterization, it became apparent that these calmodulin-enriched fractions contained, in addition to calmodulin, two additional factors with differential effects on cellular proliferation and even connective tissue production (see Figures 19 and 20). These factors have molecular weights very close to that of calmodulin and hence were not separated out by gel filtration alone. An interesting finding here was that calmodulin, purified from the lung extracts by fluphenazine-sepharose affinity chromatography,
stimulated DNA synthesis in the lung mince cultures. It was thus apparent that there were some inherent calmodulin-dependent stimulatory activities on DNA synthesis present in rat lungs, but these effects were apparently limited by heat-stable 17000-25000 dalton inhibitors. It is particularly noteworthy that calmodulin has been shown to stimulate DNA synthesis by liver cells in culture (61) and the possible involvements of calmodulin in the growth of other tissues (62,63) have been demonstrated.

The fluphenazine-sepharose affinity-purified calmodulin from the calmodulin-enriched lung fractions did not show any effect on connective tissue production. This was surprising since calcium ions have been shown to influence collagen synthesis by human skin fibroblasts in culture (143), and calmodulin is known to mediate calcium-dependent functions (57). Apparently, the heat-stable suppressive activities on connective tissue production that were present in the calmodulin-enriched fractions appeared to reside in 13700-17000 dalton regulatory proteins that are uniquely different from calmodulin but probably associated with it (see Figure 20). It is likely that the reduced activity of this protein in association with that of calmodulin in the lungs of the untreated diabetic animals may have contributed to the increased lung deposition of collagen and elastin observed in the present study. This suggestion is supported by the finding that the calmodulin-enriched fractions of soluble lung extracts (heat-treated extracts, whether DEAE-treated or not, and fraction III of Sephadex G-75 chromatography) of the untreated diabetic animals had lower suppressive effects on
hydroxyproline production compared to those of the control animals (see Figures 16 and 18). Moreover, the reports of two independent studies (153,154) suggest that calmodulin activities may be functionally relevant in connective tissue degradation by neutral proteases of the polymorphonuclear cells.

Since the lungs, from which the regulatory factors were obtained in the present study, had undergone extensive blood vessel perfusion and thus were relatively blood-free, it is believed that these factors are derived from the lung tissue but may not all be necessarily unique to the organ. Outstanding in this regard is calmodulin, a ubiquitous protein that appears to be the regulatory "corner stone" of all eukaryotic cells. However, the cellular sources of the other endogenous regulators of cellular proliferation and connective tissue production demonstrated in the present study are not known. Various studies (142,144-147) have described macrophage-derived factors which regulate fibroblast proliferation and collagen production in vitro. The study by Bitterman et al (144) demonstrated the presence of an alveolar macrophage-derived growth factor which eluted from DEAE-cellulose at 0.27 M NaCl at neutral pH and had an apparent molecular weight of 18000. The alveolar macrophage-derived suppressive factor for fibroblast proliferation and collagen production described by Clark et al (142) had an apparent molecular weight of 20000-30000 and was heat-stable. Since these factors have properties that suggest their similarity with those of the endogenous regulators described in the
present study, it is tempting to speculate that the alveolar macrophages may as well be the source of the cyclic AMP-associated regulators of cellular proliferation and connective tissue found in the lung tissues. Perhaps differences in the extent of alveolar macrophage content and its stimulation in undernourishment and in experimental diabetes may be responsible for the differences in activities observed with the lung extracts of the undernourished control and untreated diabetic animals.

H. Recapitulation and Conclusions

Although the absolute values of lung size were reduced in the untreated diabetic animals, the values normalized for body weight (the specific values) were increased and this was apparently due the specific increase in collagen and elastin contents of the lung. Cell number was reduced. The increase in connective tissue protein was concomitant with increased alveoli and internal complexity of the lung, supporting the hypothesis that elastin and possibly collagen are the major determinants of alveolar formation and maturation. Cellular proliferation was reduced in these lungs and this seemed to be due to reduced calmodulin activity, possibly resulting from enhanced activity of a 17000-25000 dalton inhibitory protein. Another lung factor (13700-17000 dalton), possibly associated with calmodulin, which suppressed connective tissue production in the control animals, was relatively unaffected by diabetes. It is likely that the sparing effect of diabetes on this regulatory protein may have caused a greater deposition of elastin and collagen, culminating in increased alveolar formation in the lungs of the diabetic animals.
The insulin-treated diabetic animals provided two important observations. Firstly, insulin therapy normalized the diabetes-induced reduction in cellular proliferation and cell number without affecting the increased connective tissue deposition. Concomitant with these effects, the diabetes-induced increase in alveolar number was not rectified, and thus indicated that connective tissue deposition rather than cellular proliferation was more relevant in alveolar formation and maturation. Secondly, since insulin treatment appeared to rectify the diabetes-induced changes in activities of the endogenous modulators, it is likely that the effects of circulating factors such as insulin, somatomedin and growth hormone on the postnatal lung may be mediated by endogenous factors such as calmodulin.

In undernourishment, cellular proliferation and connective tissue deposition in the lungs were depressed and small amount of lung tissue was thus eminent. Concomitantly, alveoli were not properly formed and thus the decreased complexity of the internal structure of the lung in the undernourished control animals. However, in undernourishment (unlike the situation in experimental diabetes), increased activity of the lung adenylate cyclase cytosolic modulator seemed to be the regulatory determinant of the suppressed deposition of connective tissue and the reduced alveolar formation. Alterations in activities of endogenous calmodulin did not seem to be of significant importance in the altered biochemistry and structure of the lung in the undernourished control animals.
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