EFFECTS OF STREPTOZOTOCIN - INDUCED DIABETES ON THE CYTOPLASMIC REGULATION OF ADENYLATE CYCLASE AND CYCLIC AMP-PHOSPHODIESTERASE IN RELATION TO THE RAT LUNG ALVEOLAR TISSUE METABOLISM

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By

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To my mother

whose love for her children has inspired in us the pursuit of knowledge as a basis of happiness.

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ABSTRACT

Some aspects of rat lung alveolar metabolism during diabetes have been investigated. Total protein content was unaffected but the DNA concentration was lowered. The proten/DNA ratio was markedly increased, suggesting increased differentiation. Hydroxyproline content was increased. Glycogen content was depleted. There was a marked reduction in the phospholipid content of the tissues and of the surfactant complex. The amount of phosphatidylcholine did not change appreciably, but the contents of disaturated phosphatidylcholine and lyso-phosphatidylcholine were markedly depleted in the surfactant complex. Also there was a reduced amount of phosphatidylglycerol in the surfactant complex. However, there was no appreciable change in the phospholipid content in the residual fractions. Ultrastructural studies showed alterations in the alveolar Type II cells which implied that the depressed metabolic activity of these cells were due to defects in the normal function of the granular endoplasmic reticulum and of the mitochondria.

Cyclic AMP and calmodulin play vital roles in cellular regulation and metabolism. In order to elucidate the role of cyclic AMP and calcodulin in the changes observed in the meta-

bolic activities of the rat lung alveolar tissue, the effects of streptozotocin-induced diabetes on the cytoplasmic regulation of adenylate cyclase and cyclic AMP phosphodiesterase were investigated. Alveolar tissues were obtained from the peripheral areas of lungs, homogenized and centrifuged at high speed to isolate a particulate fraction rich in adenylate cyclase and cyclic AMP phosphodiesterase and a supernatant fraction also rich in cyclic AMP phosphodiesterase but contained the cytoplasmic activator(s) of the particulate adenylate cyclase. Basal adenylate cyclase activity in the particulate fraction was decreased, but its activation by the supernatant fraction was markedly increased. The decrease in basal adenylate cyclase was found to be due to a translocation of calmodulin, on which it appears to depend in the expression of its activity, into the cytoplasm. The activation of particulate adenylate cyclase by the supernatant fraction appeared to be independent of calmodulin in the supernatant fraction, but was found to be due to the increased amount of a 65,000 dalton protein. Cyclic AMP phosphodiesterase activity was depressed, both in the particulate and supernatant fractions. Calmodulin translocation from the particulate into the supernatant fraction accounted for the depressed activity of the Ca^{2+}-dependent cyclic AMP phosphodiesterase in the particulate fraction. A heat-stable inhibitor of Ca^{2+}-activatable cyclic AMP phosphodiesterase was observed in the lung alveolar tissue and the activity

increased during diabetes, thus accounted for the reduced activity of the Ca^{2+}-dependent cyclic AMP phosphodiesterase in the diabetic lung alveolar tissue homogenates.

It was concluded that the altered and/or uncoordinated activities of the enzymes and the endogenous modulators related to the cyclic AMP metabolism and of calmodulin may be the most significant factors responsible for the depressed metabolic activity of the rat lung alveolar tissue during diabetes mellitus.

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A. INTRODUCTION

The ability of any organ to maintain its integrity and uniqueness depends on the differentiated properties of its constituent cells[1]. The lung is no different; however, like any other organ or tissue of an animal, it has its own characteristic pattern of biochemical activities which is associated with its physiological functions and morphological features[2]. These biochemical activities can define the altered status of lung in disease[3]. In this approach, lung disease is viewed as a process which alters lung morphology and function either directly or through the mechanisms that maintain the functional state of the lung.

It has become increasingly apparent that diabetes mellitus adversely affects the mechanical and biochemical functions of the lung [4-10]. Specific receptors for insulin have been identified in membrane preparations from normal rat lungs[8]. These studies suggest that insulin may play a role in the metabolic processes of the lung. Based on extensive information concerning the role of cyclic AMP in the anabolic action of insulin in other tissues and homogeneous populations of cells[11], it would be surprising indeed if enzymes and endogeneous modulators related to the metabolism of the cyclic nucleotide in lung tissue did not play important regulatory roles in many aspects of the depressed pulmonary activity in

diabetes mellitus.

There is no better way to appreciate what insulin means to the metabolic processes which maintain lung structure and function than to study the effect of acute insulin deprivation. It therefore becomes necessary to study the metabolic dysfunctions of the lung that affect the maintenance of the alveolar structure and function, and to examine possible alterations in the cellular mechanism(s) regulating the lung functions.

B. LITERATURE REVIEW

1. MAINTENANCE OF ALVEOLAR STRUCTURE AND FUNCTION

The major function of the lung is to provide the living organism with oxygen from the air and to remove excess carbon dioxide from the bloodstream. To accomplish this, the lung has evolved as a complex structure which brings together the atmosphere and blood in a fashion which is finely regulated to insure maximum gaseous exchange. The ability of the lung to continue to function normally in this role critically depends on the inherent properties of its parenchymal cells[1].

The mature adult lung parenchyma consists of four major types of cells (endothelial, mesenchymal cells, alveolar Type I, and alveolar Type II cells) and a complex extracellular matrix composed of three categories of connective tissue (collagen, elastic fibres, and proteoglycans). The most prevalent parenchymal cells are the endothelial and mesenchymal cells, with the alveolar Type II cells next in total number. The alveolar Type I cells are the least in number, even though the vast majority of the alveolar surface area is lined by a continuous layer of these extremely flattened and distended squamous cells with a very rich capillary bed underneath. Between these squamous superficial cells at sporadic intervals are the cuboidal shaped Type II cells, and both together with

the capillary endothelium and the reticulin basement membranes form the blood-air barrier where effective gas exchange takes place. Collagen is the most abundant constituent of the extracellular matrix, comprising 60-65% of the total extracellular mass[1].

For the lung parenchyma to maintain its function, its constituent cells must be able to modify their local environment. The inherent properties of the lung are capable of responding to a multitude of factors. The cells of the parenchyma can respond to changes in their environment because they possess the ability to receive the information of local changes. Examples of this are the receptors for corticosteroids[12,13] and the cyclic nucleotide system[14,15]. In addition, local cell-to-cell interactions can modulate changes in the cells present in the parenchyma. For example, mediators produced by parenchymal mast cells may be important in the control of smooth muscle cells located around the alveolar duct[16]. Also the demonstration of nerve endings in the region of alveolar epithelial cells suggests the possibility that direct neural control may influence the rate of surfactant synthesis and secretion[3].

The ability of the Type II alveolar cells to synthesize, store and secrete surfactant[17,18] represent a classic example of the inherent ability of a lung parenchymal cell to

modify its local environment and those of neighbouring cells. Surfactant, a surface active material which is mainly composed of phospholipids and small amounts of proteins and carbohydrates, lowers the surface tension of the alveoli, stabilizes the air spaces, and enables the lung to retain air at low inflational pressures, thus preventing alveolar collapse during expiration and greatly reducing the inspirational force required to expand the lungs[19]. Alveolar collapse occurs at birth in children with inadequate synthesis and secretion of surfactant, the syndrome known as hyaline membrane disease or respiratory distress syndrome which is a leading cause of neonatal death in developed countries.

Another inherent property and critical secretory function of lung cells is the maintenance of the extracellular matrix of the alveolar septum. Functional studies have suggested that it is an important determinant of lung mechanics and structural stability[20]. The stability of the alveoli depends, in part, on the collagen comprising them. It has been suggested that collagen acts principally as a supporting framework for elastic tissue. In this concept, the major role of collagen would be to limit expansion. However, alterations of collagen by collagenase result in a marked increase in volume at high-distending pressures with no change in the distensibility or collapsibility in the range of normal pressures. Therefore a more likely concept is that both collagen

and elastin are important determinants of lung mechanics over most of the range of inflation; collagen produces the necessary stiffness which allows increasing recoil pressures at increasing lung volumes. The synthesis of the collagen found in the alveolus appears exclusively the function of the mesenchymal cells; however, the lung endothelial cells may synthesize basement membrane collagen[1]. It is now apparent that the alveolar septum is maintained in its normal state by several processes including the relative number of cells capable of collagen production and/or catabolism, and the modulation of the cellular control of collagen synthesis and destruction by cell-cell interactions, hormonal and humoral factors[21-23]. Abnormalities in any of these processes would apparently influence the functional characteristics of the lung. Factors external to lung which either directly or indirectly promote lung metabolic dysfunction appear to affect the maintenance of parenchymal lung collagen since lung elastic recoil is significantly decreased in young men with juvenile onset diabetes[4].

2. GLUCOSE UTILIZATION BY LUNG: ITS IMPLICATIONS IN DIABETES MELLITUS

Glucose has been shown to be an important substrate for lung tissue. In addition to serving as a precursor for lung glycogen, lactic acid, and lipids[24-27], glucose optimizes the

rates of lipid and protein synthesis in the lung[25,28,29]. It has been demonstrated that glucose is taken up by the lung in amounts comparable to those taken up by other organs and metabolized via similar pathways that have been described in other organs[29-31]. Such pathways are depicted in Fig. 1. The major fate of utilized glucose is conversion to lactate. This is of interest in that the lung is one of the most aerobic organs in the body. One possible explanation for the significant quantity of lactate production is that some lung cells, or portions of cells, are dependent upon glycolysis for ATP production[32]. However, while brain must use glucose for energy because of the characteristics of the blood-brain barrier, the lung does not utilize glucose as the major energy substrate[32]. This suggests that, in lung, fatty acids or possibly amino acids also serve this function. This is appreciable since significant amounts of glucose are converted to amino acids and protein in lung tissues[29,31].

The lung does not seem to have significant capacity to store glucose as glycogen or lipid, in contrast to liver and adipose tissue[29-32]. However, lung tissue does have an adequate pentose pathway activity. This pathway is concerned not primarily with the provision of energy, but rather with the production of pentose phosphates for DNA and RNA biosynthesis and with the generation of reducing equivalents for other pathways, such as fatty acid and phospholipid synthesis,

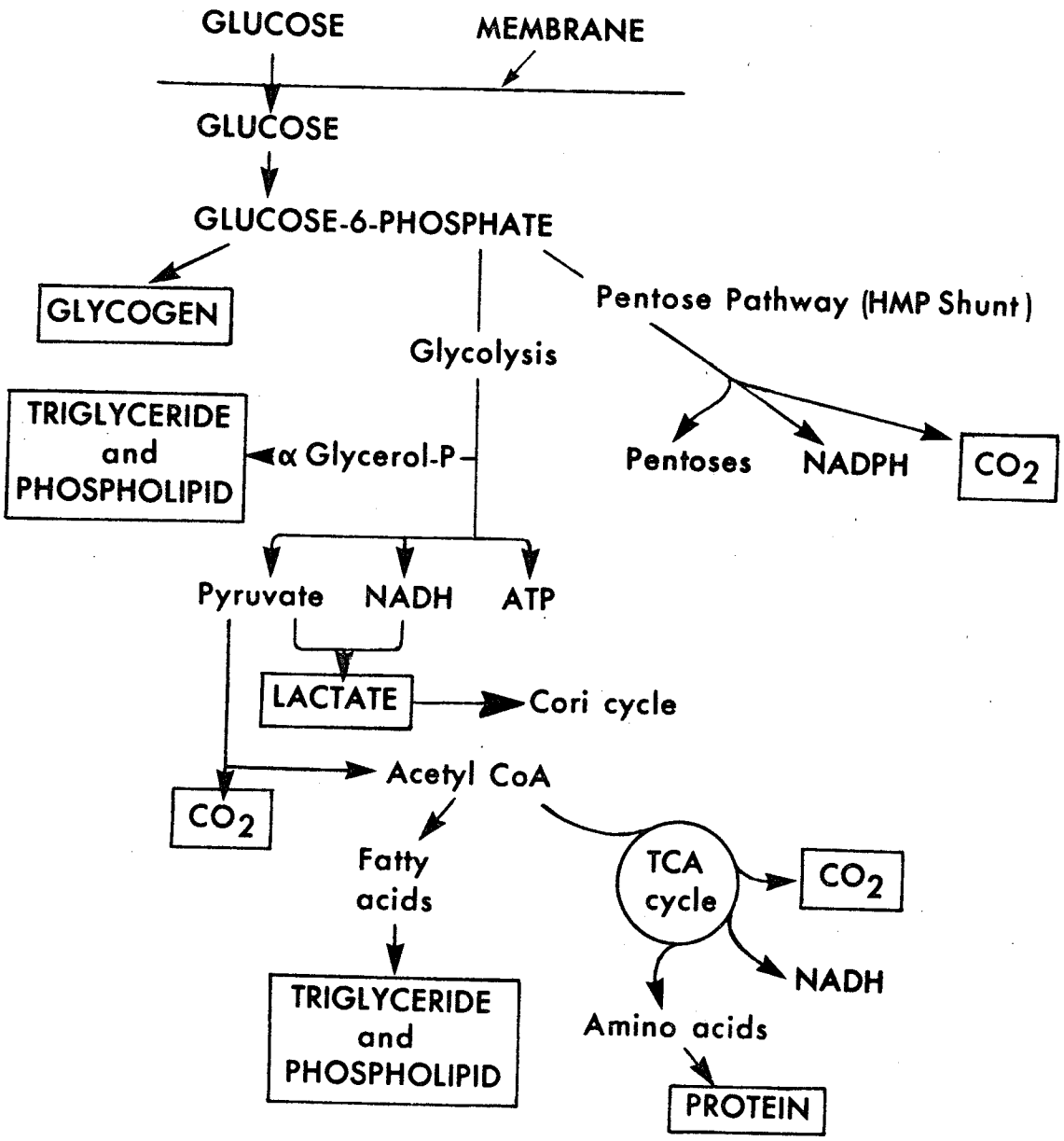


Fig. 1 Different pathways for glucose utilization

that require NADPH rather than NADH as an essential cofactor. Moreover, NADPH in some tissues, especially the erythrocyte, is essential to maintain glutathione in a reduced state and to prevent harmful oxidation, such as lipid peroxidation. It is possible that NADPH production in the lung may help to protect it from oxidants, but to date the evidence is indirect[32].

The effect of insulin on glucose transport into cells was elegantly demonstrated by Levine et al[33]. Subsequent investigations[34] indicated that glucose transport is enhanced by insulin. Recent interests have therefore focused on the possibility that glucose transport and metabolism in the lung is hormonally regulated. It has been suggested that the lung is freely permeable to glucose[24] since the addition of insulin to lung slices in vitro failed to stimulate glucose utilization. On the other hand, Weber and Visscher[26] have demonstrated an increased glucose utilization and lactate production in the presence of insulin. Morishige et al[8] also found that in the diabetic rat lung, glucose oxidation was markedly decreased and was restored to normal values with insulin treatment. Lactate production by diabetic lung slices in vitro was found to be significantly elevated, suggesting that there is a reduced flow along the pathways leading from pyruvate. The activity of the pentose pathway, which is the major pathway of glucose oxidation in lung tissue[25] was normal in the diabetic lung, suggesting that the intracellular

supply of substrate was limiting. These investigators also showed that insulin interacted in a specific manner with receptors in a particulate lung preparation. Fricke and Longmore[35] have now demonstrated that the rat lung contains a hexose transport system that is stimulated by insulin and depressed in diabetes. The transport system has several features which strongly suggest the presence of a carrier-mediated transport process, which includes an uptake process that follows Michaelis-Menten type kinetics with saturation at high substrate levels.

Moxley and Longmore[36] have shown that glucose incorporation into lipids was increased by insulin treatment and decreased in experimental diabetes. This finding is very significant since it has been suggested[37] that lung glycogen plays a role in phospholipid synthesis. Fig. 2 shows the metabolic pathways by which glycogen or glucose may be incorporated into both the glycerol and fatty acid portions of the phospholipid molecule. Pulmonary glycogen levels vary considerably during tissue development[38-40]. Accumulation of glycogen is abundant in the undifferentiated cuboidal cells throughout most of the gestation period, depleting rapidly around birth, then accumulating again by the fifth day postpartum to an adult level. The postnatal accumulation, however, is in the mesenchymal cells instead of the epithelial cells and correlates to a rapid mitotic activity of the mesenchymal

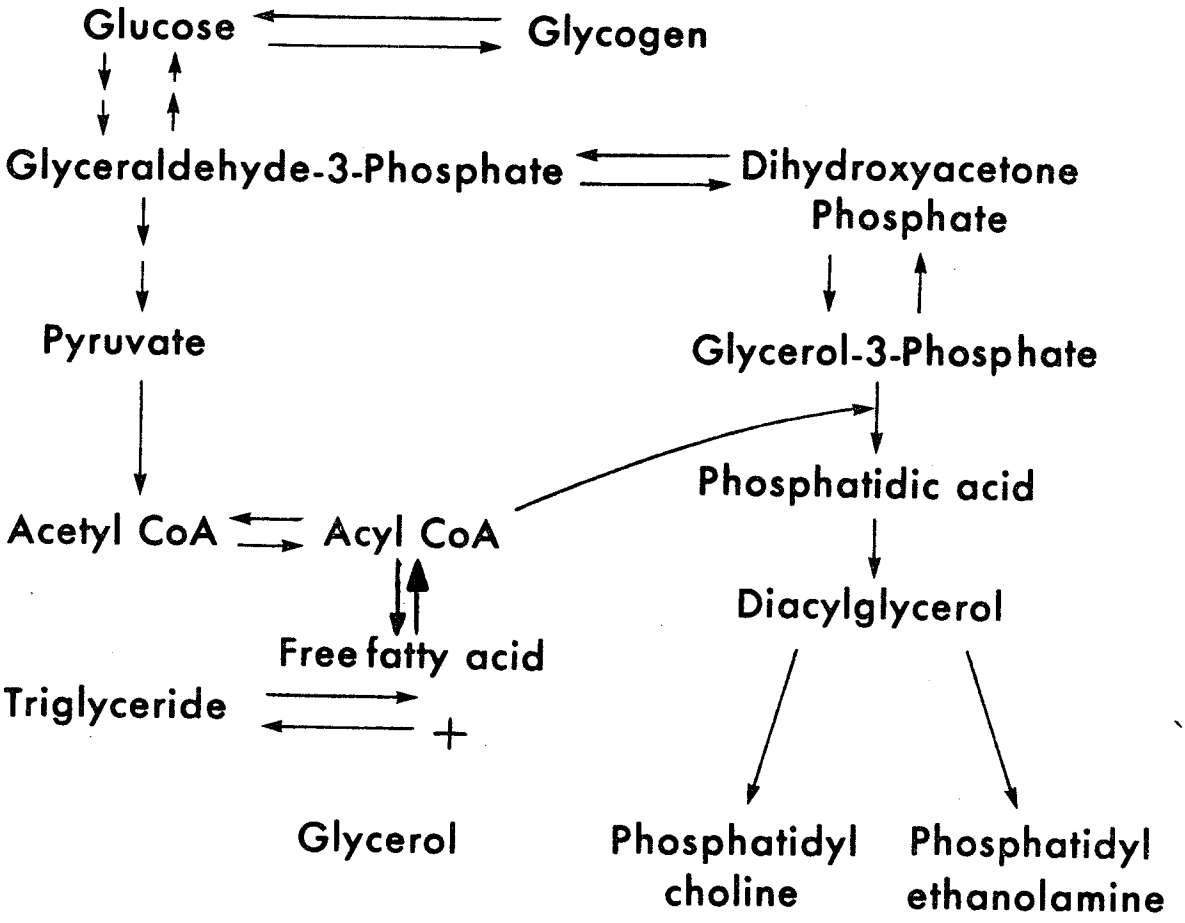


Fig. 2 Scheme by which glucose can be converted into phosphatidyl choline. Single arrowhead indicates one-step reactions and double arrow heads indicate multiple step reactions.

tissue[41]. Thus it has been suggested that glycogen provides energy for rapid cellular multiplication; the glycogen accumulating during developmental periods when there is rapid cellular multiplication and depleting when differentiation occurs. The rapid prenatal fall in fetal lung glycogen content is coincident with the differentiation of epithelial cells, and increase in pulmonary phospholipid content, and the appearance of lamellar bodies in the Type II cells[42,43]. The neonatal depletion of glycogen in lungs is apparently due to an enhanced glycogen phosphorylase a activity[38,40] and appears to provide the substrate for phospholipid synthesis[44]. It has been suggested that insulin may play a role in the short term regulation of the incorporation of glucose into the mammalian pulmonary surfactant complex[36].

3. PULMONARY LIPID METABOLISM:-- SURFACTANT PRODUCTION

It has become increasingly apparent that lipid metabolism in the lung is of considerable importance in maintaining the structural and functional integrity of the normal alveoli. The alveoli in the lung are lined by surfactant, a surface-active material, whose major components are dipalmitoylphosphatidylcholine (dipalmitoyl-PC) and phosphatidylglycerol (PG). These phospholipids are relatively rare in mammalian systems. The lung is, therefore, rather unusual in containing relatively large amounts of these two phospholipids and it

might as well contain a unique system for their synthesis[45].

Like virtually all naturally occurring phospholipids, lung phospholipid synthesis requires fatty acids and glycerol-3-phosphate or dihydroxy-acetone phosphate. The glycerol-3-phosphate (the backbone of the phospholipid molecule) may arise either from glucose via dihydroxyacetone phosphate, which is formed as an intermediate in the glycolytic breakdown of glucose, or from glycerol[37]. Under normal circumstances, the uptake of free fatty acids from the circulation is probably a major source of fatty acid for the lung. The uptake of palmitic acid from the bloodstream by the lung was shown many years ago[46]. A second external source of fatty acid is represented by circulating lipoproteins, either very low density lipoproteins synthesized in the liver or chylomicrons originating from the intestine[46]. Lipoprotein lipase has been found in the lung[47], presumably on the capillary wall, but the enzyme could also function outside the pulmonary circulation to hydrolyze circulating triglycerides to provide fatty acids to be used by the lung. The uptake of fatty acids is probably not rate limiting nor selective, but is dependent on the concentration of fatty acids in the perfusate and is altered by the distribution of pulmonary blood flow[48,49]. The lung also has a great potential for fatty acid synthesis[50]. Acetyl-CoA carboxylase and fatty acid synthetase, two enzyme systems involved in the de novo synthesis of fatty acids, are both present in the lung cytosol[51].

The major product of lipogenesis in the cytosol appears to be palmitic acid while the mitochondria possess the capacity to elongate fatty acids already formed.

Fig. 3 shows inclusively the pathways utilized in the synthesis of the major surface-active components of the surfactant. The first step in the de novo synthesis of phospholipids is the sequential double acylation of glycerol-3-phosphate on the C-1 and C-2 position to form phosphatidic acid. Phosphatidic acid is dephosphorylated by phosphatidase phosphatase to form 1,2-diacylglycerol which reacts with CDPethanolamine to form PC or PE in reactions catalyzed by cholinephosphotransferase or ethanolaminephophotransferase, respectively[52]. The PE may be converted into PC by three sequential N-methylation steps catalyzed by phosphatidylethanolamine methyltransferase[53]. Phosphatidic acid also reacts with CTP to form CDPdiacylglycerol which reacts with a second molecule of glycerol-3-phosphate to form PG-phosphate in a reaction catalyzed by glycerolphosphate phosphatidyltransferase. PG-phosphate does not accumulate but is immediately dephosphorylated to PG[54].

In the lung, the above described CDP- choline pathway represents the major route involved in the synthesis of unsaturated PC molecules. However, there is substantial evidence[54] that the lung has other mechanisms that accomplish

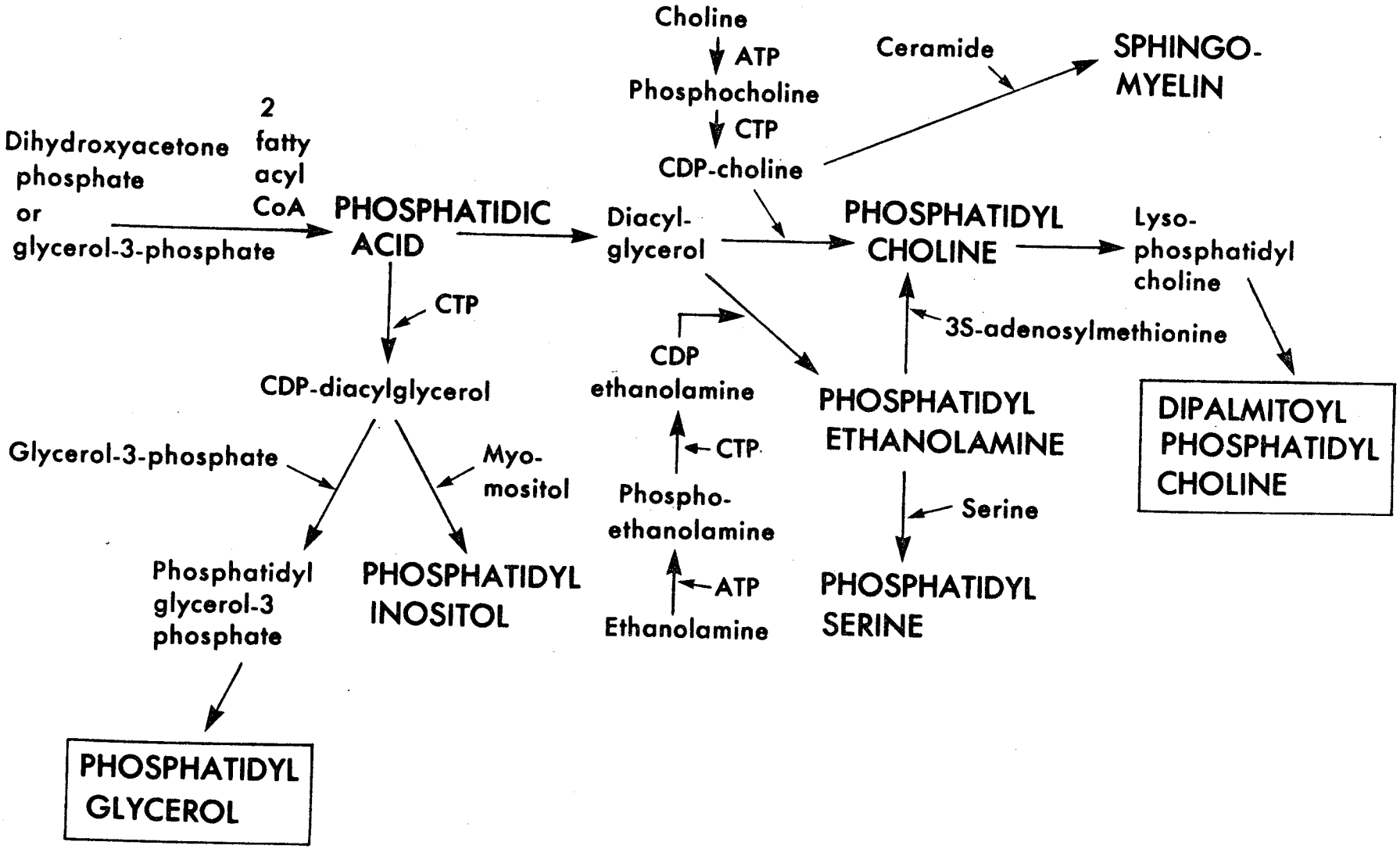


Fig. 3 Pathways of Phospholipid Synthesis

the remodeling of de novo synthesized unsaturated PC into dipalmitoyl-PC. Two auxillary mechanisms are involved in this transformation and they comprise of a deacylation-reacylation process with 1-palmitoyl lysoPC as an intermediate metabolite. It is important to stress that these auxillary mechanisms cannot lead to a net increase of the total PC pool but can only accomplish a shift in the molecular composition of the total PC fraction.

The controversial involvement of Clara cells in the secretion of surfactant[55-57] has now given way to the quite certain bulk of evidence that pulmonary alveolar Type II cells are the sites of surfactant production[42,58,59]. The phospholipids are synthesized in the endoplasmic reticulum of the epithelial cells, packaged in the golgi apparatus and stored in the lamellar bodies in the form of lipoprotein[60,61]. The association of the phospholipids of surfactant with certain apoproteins has been postulated to accelerate the extracellular transport of pulmonary surfactant and to insure that adequate amounts of the surface-active maerial are available to the alveolar surface[62]. Secretion of surfactant appears to be controlled by both adrenergic[63] and cholinergic[64] mechanisms. The role of lamellar bodies in the biosynthesis of surfactant is not clear at present; there are evidence both for[59,65,66] and against[67,68] it. Garcia et al[68] and Longmore et al[69] have reported evidence of a Ca^{2+}-dependent

phospholipase A_2 activity in the rat lung microsomal fractions but not in the lamellar bodies. This finding is in conflict with the recent report of Heath and Jacobson[70] that lysosomal forms of phospholipases A_1 and A_2, both inhibited by Ca^{2+}, are present in the lamellar bodies. The lysosomal phospholipase A_2 in particular may be concerned with the remodeling of unsaturated PC into the dipalmitoyl species. A failure in the activity of this enzyme may as well be the basis for the respiratory distress syndrome in the newborn[70].

4. PULMONARY LIPIDS IN DIABETES MELLITUS

A major cause of mortality in the neonatal period is respiratory distress syndrome[19]. This disease is characterized by biochemical alterations and a deficiency in lung surfactant. Evidence indicates that the respiratory distress syndrome in infants from diabetic mothers is six times more than that from non-diabetic mothers[6]. The demonstration by Rhoades et al[71] that the percent of total phospholipid made up of PC was not altered while the percentage of disaturated PC was decreased in rat lungs of neonates from diabetic mothers is particularly important and assumes functional significance since the disaturated PC is the principal agent responsible for surface tension reducing properties in the surfactant complex[19]. It appears that the deficiency of other components of surfactant such as PG may also be responsible for the develop-

ment of the respiratory distress syndrome[72,73]. The activity of the CDPcholine pathway enzymes showed a marked increase in lungs of neonates from diabetic mothers. These observations suggested that pathways other than those responsible for the first step in phospholipid synthesis may be affected such as the deacylation and reacylation enzymes, lysoPC acyltransferase and lysoPC:lysoPC acyltransferase[71]. The pattern of glucose utilization in the neonatal lung was also altered. Particularly significant was the observation that glucose oxidation to CO_2 and the incorporation of glucose into neutral lipids decreased while glucose incorporation into lung phospholipid was unchanged. Thus the investiagors[71] suggested that glucose was being conserved for glycerol-3-phosphate which was then used in phospholipid synthesis. However, glycoven in lungs from neonates of the diabetic mothers showed a significant increase. Neufeld et al[74] have presented a possible mechanism for the dysfunction. High maternal glucose crosses the placenta, stimulates insulin release by the fetal pancreas and, in some way, the insulin inhibits lung glycogenolysis as it does in the liver and, thus, diminish availability of carbohydrate for surfactant synthesis. The marked decrease in protein/DNA ratio, coupled with increased DNA levels, suggested the presence of more undifferentiated lung cells in the neonates from diabetic mothers[71].

The results of investigations with experimental diabetes

in adult animal models have provided insights into the effects of insulin deficiency on lung lipid metabolism. Das and Kumar[5] have reported that streptozotocin-induced diabetes lowered the lipid-synthesizing capabilities of rat lung homogenates by altering the levels of acetyl-CoA carboxylase and fatty acid synthetase. Moxley and Longmore[7] have demonstrated that experimentally induced diabetes decreases the incorporation of glucose into lung lipids. Effects of experimental diabetes on the lipid components of the surfactant and residual fractions of the lung were also examined[36]. The investigators demonstrated decreased glucose incorporation into the residual fractions and more importantly into the surfactant lipids of the lung; also decreased glucose incorporation into PC and PG. The finding that glucose incorporation into the fatty acid moiety and the glycerophosphocholine moiety of PC was decreased in diabetic lungs indicated that pathways other than those responsible for fatty acid synthesis are affected.

Studies of plasma lipids and lipoproteins in diabetes have shown marked increase in these substrates which contribute appreciably toward lipogenesis[75]. Pathak et al[76] have reported a twofold increase in activity of lipoprotein lipase in the diabetic lung. The increase in activity may be either due to conversion of inactive form of the enzyme to its active form as has been reported in other tissues[77] or due to the

induction of enzyme by increased levels of circulating triglycerides, especially in the form of very low density lipoproteins[78]. The enhanced lipoprotein lipase activity may be responsible for an increased supply of free fatty acids available for de novo synthesis of triglyceride, which possibly could account for the increased triglyceride content in acute[10] and chronic diabetic lung[76]. The increased triglyceride content, while phospholipids and cholesterol decreased in diabetes when compared to the control group, thus resulted in no change in total lung lipids[10,76]. The activity of hepatic B-hydroxy-B-methylglutaryl-CoA (HMG-CoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis, has been reported to be reduced in diabetes[79,80].

5. LUNG PROTEINS AND INFLUENCE OF CONNECTIVE TISSUE PROTEINS IN DIABETES

It is not surprising that an organ as complex as the lung is composed of a large number of diverse proteins. Some of these, such as the enzymes of the intermediary metabolism, are proteins present in cells throughout the body and are necessary for general cellular function. Other proteins, such as the apoprotein of surfactant, are probably specific for the lung. Still others, such as the structural proteins of the connective tissue, are present in other organs but not necessarily in the same relative amounts as found in the lung[81].

The presence of these proteins depends upon the complex protein synthesizing machinery found in each of the approximately forty different cell types of the lung. Each cell type can be expected to synthesize a set of proteins common to all cells but, in addition, the fact that cells are different means they must synthesize different amounts, and probably diverse types, of proteins. For example, both the alveolar macrophage and the alveolar Type II cell synthesize the enzymes involved in lipid metabolism but only the Type II cell synthesizes the apoprotein of surfactant. Likewise, both the fibroblast and the chondroblast synthesize the enzymes required for glycolysis, yet the fibroblast synthesizes Types I and III collagen and the chondroblast probably synthesizes only Type II collagen[81].

The biochemical basis of lung function can be defined in terms of the averaged expression of the differentiated state of all lung cells, and the expression of this differentiated state connotes the presence at any one time of the functional proteins which comprise each cell[81]. The presence of proteins found in every organ is controlled by a balance between protein synthesis and degradation[82]. There is an increasing appreciation of the possibility that the process of protein degradation may have additional physiological significance. For example, it may play a role in the removal of abnormal, potentially harmful proteins from a cell,

and may increase in poor or altered environments to provide amino acids so the cell can synthesize new enzymes or other proteins appropriate to the new conditions[83].

Thet et al[84] have reported that insulin appears to influence protein metabolism in the lung by increasing amino acid uptake and by decreasing protein degradation. Also glucose does not alter protein synthesis in the lung, but rather decreases proteolysis. However, glucose and insulin together are not synergistic in their proteolytic actions. Chiang and Massaro[85] have examined the effect of exogenous amino acids and of exogenous glucose on protein degradation in the rat lung. The absence of exogenous amino acids lowered the rate of proteolysis of rapidly but not of slowly turning-over proteins. Addition of normal rat plasma levels of amino acids returned the rate of proteolysis to control levels. Exogenous glucose influenced the degradation of slowly but not of rapidly turning-over proteins. Thus it appears that the degradation of rapidly and slowly turning-over proteins are independently regulated in the lung.

Enzymes form a special group of proteins whose activities have been measured in studies of experimental short-term diabetes. In most cases, the reversal of the diabetic changes by insulin have been blocked by inhibitors of protein synthesis. Thus it appears that most changes in activity are

in fact changes in amount of protein[86]. However, the demonstration that lung elastic recoil is significantly decreased in young men with juvenile onset diabetes[4] has focused the interests of investigators in connective tissue proteins of the lung. Biochemical and morphological studies of the connective tissue components of various organs and tissues indicate that in diabetics, collagen[87,88], elastin[89], and basement membrane[90,91] undergo changes analogous to those which occur during aging. Abnormalities in protein and collagen metabolism of fibroblasts from juvenile-onset and adult-onset diabetics have also been described[92]. Madia et al[93] demonstrated a higher response in acetic acid-soluble hydroxyproline which suggested an increase in acid-soluble collagen synthesis in the lung in response to the induction of the diabetic state. The activity of lysyl oxidase, the enzyme which generates the cross-linkage precursors, was elevated 2-3 fold. It was suggested that the responsiveness of the enzyme in the lung tissue to diabetes may be a component of a more generalized response in collagen and elastin synthesis which eventually may contribute to alterations in the functional properties of the lung. Vracko et al[94] have reported that the amounts of epithelial and capillary basement membrane in alveolar walls were increased in patients with diabetes mellitus; however, the extent of basement membrane thickening in the lung was relatively small. Basement membrane thickening did not seem to be a prominent

feature in the lung of diabetics and, if present at all, was probably compensated functionally[95]. Ultrastructural studies[96,97] have not demonstrated any basement membrane changes in streptozotocin-induced diabetes in the rat lung; however, they do show alterations in the alveolar Type II cells and in nonciliated bronchiolar secretory epithelial (Clara) cells which represent a depression of their biosynthetic activity and therefore changes in pulmonary metabolic functions associated with diabetes mellitus.

6. CYCLIC AMP METABOLISM: ENZYMES AND MODULATORS

(i) Adenylate Cyclase: General Properties and Regulation---

Adenylate cyclase, the enzyme that produces cyclic AMP, is part of a complex regulatory system that mediates the actions of hormones and neurotransmitters on their target cells. Adenylate cyclase is associated with the cell membrane; the enzyme system is believed to harbour a hormone receptor site facing the extracellular space and a catalytic site facing the cytoplasmic space. Stimulation of the enzyme leads to an increase in cyclic AMP, which mediates the effect of the hormones[98]. Although adenylate cyclase in many types of cells is sensitive to a variety of hormones it is generally believed that only a single adenylate cyclase system is in-

volved. The differences of hormonal response in different cells resides in the presence of distinct receptors for specific hormones[99].

Ions appear to play a vital role in the regulation of adenylate cyclase activity. Almost all of the adenylate cyclase systems described thus far in eukaryotic cells have been found to be stimulated by fluoride ion[100]. Adenylate cyclase activity has an absolute requirement for Mg^{2+} ions[101]. The true substrate for the enzyme is Mg^{2+}-ATP and free ATP is inhibitory to the reaction[101,102]. It has now been suggested that in addition to the catalytic site there may be a second allosteric site for the Mg^{2+} ions whose occupation necessitates the expression of catalytic activity[99]. Calcium ion, in micromolar concentrations, has been shown to be essential for basal adenylate cyclase activity and its hormonal stimulation but higher concentrations were invariably found to be inhibitory[103,104].

There has been recent interest in the observations that cytoplasmic factors acting on the inner surface of the plasma membrane may also be important in regulating the activity of adenylate cyclase. GTP has been shown to affect the activity of the cyclase and to modulate the response of the enzyme to hormones[105-107]. Hegstrand et al[108], however, have now demonstrated that the effects of this naturally occurring

purine nucleotide on binding of agonists to beta adrenergic receptors, on basal adenylate cyclase activity and on the coupling of the beta adrenergic receptor to adenylate cyclase appear to occur independently. Doberska and Martin[109] and Pecker and Hanoune[110] have reported, independently, the presence of a heat-stable cytosolic protein factor which modulated the basal and hormonally-stimulated adenylate cyclase activity in rat liver. The factor appeared to act like GTP and the findings that GTP and cytosolic factor did not produce an additive activation of the enzyme suggested a common site of action[109]. Sanders et al[111] described the presence of a protein-like activator of adenylate cyclase in the 10,000xg supernatant of heart and other tissues. The factor was heat labile, non-dialyzable, sensitive to trypsin digestion and had no effect on the basal adenylate cyclase activity but enhanced the cyclase activation by epinephrine and glucagon (not by NaF). Katz et al[112] reported a cytosol protein activator which restored the basal and the epinephrine, glucagon, and flouride activation of adenylate cyclase in the particulate fraction prepared from rat liver. Beaumont et al[113] have given evidence to demonstrate the presence in erythroid cell cytoplasm from several species of non-dialyzable soluble factors capable to activate basal adenylate cyclase and to modulate the action of positive effectors. The results suggested that GTP was probably not responsible for the observed stimulation of the cyclase.

The ubiquitous heat stable Ca^{2+}-dependent regulatory protein (Calmodulin) has been established to mediate the activation of mammalian brain adenylate cyclase by Ca^{2+}[114,115]. Adenylate cyclase activities stimulated by dopamine[116], GTP[117] and cholera toxin[119] are also activated by Ca^{2+} and calmodulin. Nijjar[120] has given evidence of the presence of some stimulatory factor(s) in the 105,000xg supernatant fraction from rat brain which markedly enhance the activity of the adenylate cyclase in the particulate fraction. The cytoplasmic factor(s) produced additive activation of the enzyme with NaF but not with GTP or calmodulin. Thus, the investigator has suggested that the rat brain supernatant may contain some factor in addition to calmodulin which activates the particulate adenylate cyclase. Some tissues do contain calmodulin activity but do not appear to possess the calmodulin-dependent adenylate cyclase activity[117].

Nijjar[121] had earlier demonstrated the presence of an age-dependent cytoplasmic factor in the rat lungs which enhanced the adenylate cyclase activity in the particulate fraction. This activator appears to be a protein and did not resemble either GTP or calmodulin in its action on adenylate cyclase. The activator activity did not appear in the cytosol until around the twentieth day of age. It was therefore suggested that the activator may have a role in cellular differentiation as the time of appearance of the factor coincides

with the time of cessation of rapid alveolar multiplication in the rat lungs. Particularly important was its suggested significance as a biological regulator(s) of lung functions in vivo.

(ii) Cyclic Nucleotide Phosphodiesterase and Calmodulin---

Tissue levels, and therefore the effective concentrations, of cyclic AMP depend not only on its rate of formation but also on the rate of degradation effected by cyclic 3',5'-nucleotide phosphodiesterase (PDE). Conversion of cyclic AMP to 5' AMP is the only physiological mechanism known to terminate the action of cyclic AMP[122]. Multiple forms of PDE have been found in every tissue examined, including lung tissue[122-125]. Russel et al[126] have demonstrated that DEAE-cellulose chromatography of liver extract, prepared by homogenization, sonication, and centrifugation, exhibits three discrete active fractions of PDE activity referred to as D-I, D-II, and D-III according to their elution from the column by a salt gradient. This chromatographic analysis has been applied to a number of mammalian tissues including the lung[127] and while the relative amounts of the different forms of PDE activities vary from tissue to tissue, PDE activity was conserved.

Of the three forms of PDE activity, D-III appears to have a low K_m (cyclic AMP) particulate enzyme. The activity

of D-III is characterized by relative selectivity toward cyclic AMP as substrate and appears to be under negative cooperative control by its substrate[128]. While the D-III enzyme is particulate, the D-I and D-II forms appear to be soluble. The D-II enzyme has been identified as possessing approximately equal activity toward cyclic AMP and cyclic GMP[127]. Beavo et al[129] have demonstrated that cyclic GMP may regulate the activity of the D-II enzyme. Both the D-II and the D-III enzymes have no sensitivity to Ca^{2+} but the D-I enzyme which hydrolyses both cylic AMP and cyclic GMP (with higher affinity toward cyclic GMP) is Ca^{2+} sensitive. Independently, Teo and Wang[130] and Kakiuchi et al[131] have demonstrated that the activation of the Ca^{2+} -sensitive enzyme in bovine heart and rat brain respectively, depends on the presence of a Ca^{2+}-binding protein, originally discovered by Cheung[132] and recently renamed Calmodulin. The calmodulin exists as an independent protein molecule which associates with the enzyme only upon the binding of Ca^{2+}.

Hitchcock[124] found both a low affinity (PDE I) and a high affinity (PDE II) soluble cyclic AMP phosphodiesterase present in the guinea pig lung. Both forms required Mg^{2+} and could be inhibited by methylxanthines e.g. theophylline or caffeine. However, only PDE I was inhibited by Ca^{2+}. Interestingly, both Ca^{2+}-activated cyclic nucleotide phosphodiesterase and calmodulin activities have been demonstrated

in human and guinea pig lungs[133-135]. Sharma and Wirch[136] have recently confirmed these activities in the rabbit lung. However, the Ca^{2+}-activatable cyclic nucleotide phosphodiesterase is different from the common type in that it contains tightly bound calmodulin. This bound calmodulin is not dissociated from the enzyme even in very low concentrations of Ca^{2+}. Therefore, it appears that the calmodulin mediates the Ca^{2+} effect on the enzyme. However, the mechanism of the enzyme activation has been established in the brain as follows[137]:

$$Ca^{2+} + CM_{(inactive)} \rightleftharpoons Ca\text{-}CM^{*}_{(active)}$$

$$Ca\text{-}CM^{*} + Enz_{(inative)} \rightleftharpoons Ca\text{-}CM^{*}\text{-}Enz^{*}_{(active)}$$

where CM and Enz represent the calmodulin and the enzyme, respectively, and the asterick indicates the activated state of the protein species.

The Ca^{2+}-stimulated PDE can also be activated by proteolysis[138] and by certain lipids and phospholipids, particularly phosphatidyl inositol and lyso phosphatisyl choline[139], but this stimulation is independent of Ca^{2+}. A heat-labile protein that specifically inhibits the Ca^{2+}-activated PDE reaction has been discovered in bovine braine[140]. This brain protein, designated as the modulator-binding protein,

associates with the modulator protein in the presence of Ca^{2+}. It is present in high amounts in bovine brain but little or none of it is present in bovine heart[137]. Also, a heat-stable inhibitor protein of the Ca^{2+}-activated PDE has recently been shown to exist in bovine brain[141] and possibly other mammalian tissues[140]. On a unit-weight basis, the heat-stable inhibitor protein appears to have much higher inhibitory activity than the modulator-binding protein, suggesting that the heat-stable inhibitor protein may have a higher affinity toward the modulator[140]. However, the physiological significance of the heat-stable inhibitory protein is not known at present, but Sharma et al[142] have suggested that if the protein is indeed a physiological inhibitor, it must be restrictively localized in the tissue or its concentration in the tissue can be altered markedly under certain conditions; on the other hand, the protein may be another modulator-regulator enzyme.

(iii) Diabetes and Abnormal cyclic AMP Metabolism ---

As previously mentioned, the activities of adenylate cyclase and cyclic nucleotide phosphodiesterase determine the effective tissue concentrations of cyclic AMP. Cyclic AMP has been ascribed a fundamental regulatory function in the cells[143]. Of special relevance to the maintenance of the integrity and metabolic uniqueness of an organ or tissue are

the indications that cyclic AMP, in at least some tissues, is of central importance in maintaining the differentiated state[144]. Cyclic AMP mediates the action of many hormones in a variety of tissues[143]. The realization that calmodulin functions both as a mediator of Ca^{2+} functions and as a regulator of Ca^{2+}-dependent adenylate cyclase and phosphodiesterase provides a functional link between these two classes of intracellular regulators[145]. In many instances, the effects of the calmodulin-Ca^{2+} complex and of cyclic AMP are often intertwined, that is, one may accentuate or attenuate the effect of the other.

The diabetic state is characterized by a deficiency of insulin secretion due to a defect in the number of beta cells of the pancreas, often to less than ten percent of normal[146]. The role of cyclic AMP in insulin action has been established[11]. Insulin does not activate adenylate cyclase[147]; however, it has been postulated that at least some of the metabolic actions of insulin (that is, as the major anabolic hormone) are due to the activation of a high-affinity, cyclic AMP-specific phosphodiesterase contained in particulate fractions and perhaps associated with an adenylate cyclase and therefore able to reduce the cyclic AMP concentration in at least some intracellular pool if not in the entire cell[127]. Thompson et al[148] have demonstrated that the membrane-bound high affinity cAMP-PDE of diabetic rat liver is stimulated by insulin ad-

ministration, but the cGMP-PDE which are separate and apparently soluble enzymes were unaffected. Apparently insulin increases the PDE activity by stimulating the synthesis of the enzyme. Interestingly, Solomon et al[149] have shown that experimental diabetes decrease the level of calmodulin, thus offering an explanation for the decrease in total PDE activity. Solomon's group[150,151] have gone further in their investigations to show that in isolated liver cells during insulin deprivation, major shifts in subcellular distribution of PDE and its calmodulin regulator occur in addition to the presence of a calmodulin inhibitor protein. The investigators have therefore suggested that the redistribution of these components from the sites of physiological regulation may play a major role in the distortion of cyclic AMP metabolism observed during diabetes.

Diabetes has been described as a bihormonal disease caused not only by insulin deficiency but also by the excess of glucagon[152]. Glucagon stimulation of adenylate cyclase will increase the cyclic AMP content of the cell[147]. Several conflicting observations have been reported regarding the binding and biologic activity of glucagon in the diabetic rat liver. Independently, Soman and Felig[153] and Hepp[154] have reported increased binding of glucagon to liver plasma membrane preparations and a two-fold increase in the basal and glucagon-stimulated adenylate cyclase activities in

streptozotocin-diabetic rats. On the other hand, Pilkis et al[155] observed that glucagon-stimulated adenylate cyclase in membrane fractions were not different in the diabetic and control samples. Yamashita et al[156] have recently demonstrated that responses to glucagon of the adenylate cyclase-cAMP system in liver slices from diabetic rats are significantly lower than those in liver slices from normal rats; however, the basal and glucagon-stimulated activities of the adenylate cyclase in crude membrane fractions were similar in both groups. The reduced responsiveness to glucagon in vitro in liver slices may be explainable as a hormone-specific desensitization or refractoriness induced by the glucagon excess in diabetes[156]. Nonetheless, these observations do demonstrate abnormalities in cyclic AMP metabolism in liver tissues of the diabetic animal.

The diabetic state also appears to involve the catecholamine stimulation of adenylate cyclase. For example, Menahan et al[157] have reported a lack of epinephrine stimulation of rat heart adenylate cyclase in experimental diabetes. However, the basal activity was unchanged but sodium flouride-stimulated adenylate cyclase was lower when compared to basal activity in the diabetic groups. Nonetheless significant is the demonstration by Zumstein et al[158] that there is a significant decrease in basal adenylate cyclase activity in fat cells of diabetic rats, but an increased sensitivity to epinephrine

and isoproterenol. Thus the diabetic state may lead to increases or decreases in the cyclic AMP concentration of tissues, depending on the tissue involved.

C. OBJECTIVES OF PRESENT STUDY

Diabetes is now known to adversely affect the mechanical and biochemical functions of the lung. Morphological and biochemical studies suggest that these effects reflect a depression of metabolic activities. Specific receptors for insulin have been identified in membrane preparations from normal rat lungs. However, the cellular mechanism whereby insulin availability modulates the lung metabolic activities remains unknown. The role of cyclic AMP in the metabolic action of insulin has been established in other tissues and homogeneous populations of cells. Furthermore, cyclic AMP and calmodulin have been established to play vital roles in cellular metabolism. Hence these cellular regulators are worth studying during insulin deprivation in rat lungs.

Hypothesis: Insulin deprivation in rat lung alveolar tissue may cause alterations in the activities of Adenylate cyclase and cyclic AMP-Phosphodiesterase (PDE), both enzymes responsible for the effective concentration of tissue cyclic AMP. Recent ideas are that cytoplasmic factors regulate the activity of particulate adenylate cyclase and also that calmodulin functions as a regulator of both adenylate cyclase (in some tissues) and PDE. These regulators may be altered during diabetes. Since cyclic AMP and calmodulin play vital roles in cellular metabolism, studies of these

cellular regulatory components and of some metabolic parameters could give a clearer picture of how changes in activities of the regulatory may account for the changes in the metabolic activities of rat lung alveolar tissue during diabetes.

In the present study, the experimental model utilizes adult rats since there is a lack of spontaneous recovery from the streptozotocin-induced diabetes in adult rats in contrast to the partial recovery observed in neonatal rats. By using only the peripheral areas of the lungs, the metabolic parameters of the diabetic alveoli will be examined biochemically. Ultrastructural studies will be carried out to supplement the biochemical findings. A major part of the study will examine the effects of diabetes on the cytoplasmic regulation of adenylate cyclase and on the activity of cyclic AMP-phosphodiesterase. The activities and levels of calmodulin will be measured and compared in the lung tissue from diabetic and control rats. An attempt will be made to determine possible regulation of lung adenylate cyclase by calmodulin. Lung tissue will be examined for the inhibitory protein of Ca^{2+}-activatable phosphodiesterase and the inhibitory effect compared in diabetic and control rat lungs.

D. EXPERIMENTAL PROCEDURES

1. Experimental Animals

Adult male Sprague-Dawley rats (100-130 gm. body weight) were obtained from the Faculty of Dentistry, University of Manitoba. For induction of diabetes, rats were injected intravenously with a single dose of streptozotocin (75 mg/Kg of body weight in isotonic saline)[8]. Control animals were treated similarly by injecting isotonic saline. Four days later, a group of the streptozotocin-injected rats received daily subcutaneous injections of protamine zinc insulin (1 unit/day/rat) until termination. All the rats were left for another seven days while being maintained on standard rat chow and tap water in metabolic cages. Daily urine glucose was measured (with Eli Lilly Tes-Tape). Prior to termination of the rats, blood was collected by cardiac puncture and serum glucose determined colorimetrically (Sigma Tech. Bulletin #510, 1978). Only streptozotocin-treated rats with about 3+ urine glucose and serum glucose over 300 mg/100ml were used in the study.

2. Preparation of Lung Tissue Fractions

The animals were decapitated and exsanguinated, and the

lungs were quickly removed and washed in 0.27 M sucrose - 10 mM Tris-HCl buffer, pH 7.4 in a chilled petri dish. The lung tissues were dissected free from all visible bronchi and vasculature, and only the peripheral areas of the lungs consisting predominantly of alveolar tissues were selected for homogenization. All operations were carried out at 2-4^{o}C unless otherwise stated.

The lung tissues were sliced with a sharp razor blade and homogenized in nine volumes of the buffer in a Potter-Elvehjem homogenizer by 15 hand-driven strokes. The homogenate was then centrifuged at 15,000xg for 10 minutes in an International refrigerated centrifuge (Model B-20). The supernatant was withdrawn and centrifuged at 105,000xg for 60 minutes in a swinging-bucket rotor in an International refrigerated centrifuge (Model B-60). The supernatant was withdrawn and henceforth designated the supernatant fraction. The pellet was rehomogenized with a loose fitting homogenizer (5 strokes), recentrifuged and suspended in the homogenizing buffer to give a 100 percent suspension on the basis of original tissue weight; this fraction was henceforth designated the particulate fraction[121]. The samples were stored at -20 C until various enzyme assays were performed, usually within one week.

3. Estimation of Protein

Protein was measured by the method of Lowry et al[159] using bovine serum albumin as standard. The basic principle of this measurement is that protein forms a protein-Cu^{2+} complex in alkali, and this complex reduces the phosphomolybdic-phosphotungstic reagent to give a measurable color. An aliquot of tissue fraction was dissolved overnight in 1 ml of 1 N sodium hydroxide, and 0.2 ml aliguot of this solution was made to 0.4 ml with distilled water. The color reagent containing 0.02% (w/v) of 1 N NaOH in 2 ml of 2% (w/v) sodium carbonate was added, and the mixture was allowed to stand for 10 minutes. Then 0.2 ml of 1N Folin's reagent was added and the reaction was allowed to continue for another 30 minutes at room temperature. The absorbance was read at 750 nm.

4. Estimation of DNA Content

Samples were denatured in a 24 percent TCA solution and centrifuged at 5,000 rpm. The precipitate was suspended in 16.5 percent TCA and centrifuged again. The precipitate was then suspended in 3 ml of 5 percent TCA solution and heated at about 90°C for 10 minutes. The suspension was then centrifuged. 1 ml of the supernatant was analyzed for DNA content by adding 2 ml of 1 percent diphenylamine in glacial acetic acid plus 2.75 percent of sulfuric acid, and then

boiling the mixture for 10 minutes. The mixture was allowed to cool and the absorbance was read at 600 nm[160].

5. Estimation of Gycogen

The determination of glycogen content was carried out as described by Lo et al[161]. Tissue samples weighing about 60 mg were digested in 1.5 ml of 30 percent potassium hydroxide solution saturated with Na_2SO_4 for 30 minutes in a boiling water bath. The samples were cooled in ice and 1.63 ml of 95 percent ethanol was added to precipitate glycogen. After 30 minutes, the samples were removed from ice and centrifuged at 840xg for 30 minutes. The glycogen precipitates were then dissolved in 3 ml distilled H_2O. 200 ul aliguot of the glycogen solution was made to 1 ml with distilled H_2O and to this was added 1 ml of 5 percent phenol solution followed immediately with 5 ml of 96 percent H_2SO_4. After standing at room temperature for 10 minutes and followed by shaking in a 25-30°C water bath, the absorbance was read at 490 nm.

6. Estimation of Phospholipid Content

The procedure utilized in the study entailed the isolation of a surface-active fraction from the rat lung alveoli according to the discontinuous sucrose density centrifugation method described by Frosolono et al[162].

This surfactant fraction, unlike the surface-active fraction obtained by lavage of lung, contains the total surfactant pool of the lung, both intracellular and extracellular components. These two pools of surfactant material have been found to have a similar chemical composition and surface activity[163]. Tissue samples were homogenized in nine volumes of the homogenization medium (0.145 M NaCl in 0.01 M Tris-HCl containing 0.001 M EDTA, final pH 7.4) in a Potter-Elvehjam glass homogenizer. 2 ml of the homogenate was carefully layered over 3 ml of 0.75 M sucrose. Fig. 4 shows a flow diagram of the procedures used subsequently. The surfactant band (IB) was found between the 0.25 and 0.68 M sucrose layers. All materials not recovered in the surfactant band were pooled and now referred to as the residual fractions. Volumes of the fractions were all made to approximately 2 ml by thin film evaporation.

Lipids were extracted from the tissue homogenates and the fractions with 5 ml of chloroform:methanol (2:1, v/v) in graduated glass stoppered tubes. The solutions were allowed to stand overnight at room temperature. Then 1 ml of 0.01 N HCl was added, mixed by inversion and centrifuged for 1-2 minutes in a clinical centrifuge. The upper aqueous phase was discarded. The lower phase was washed two times with approximately 2.5 ml of chloroform:methanol: 0.01 N HCl (3:48:47, v/v), discarding the upper phase each time. The

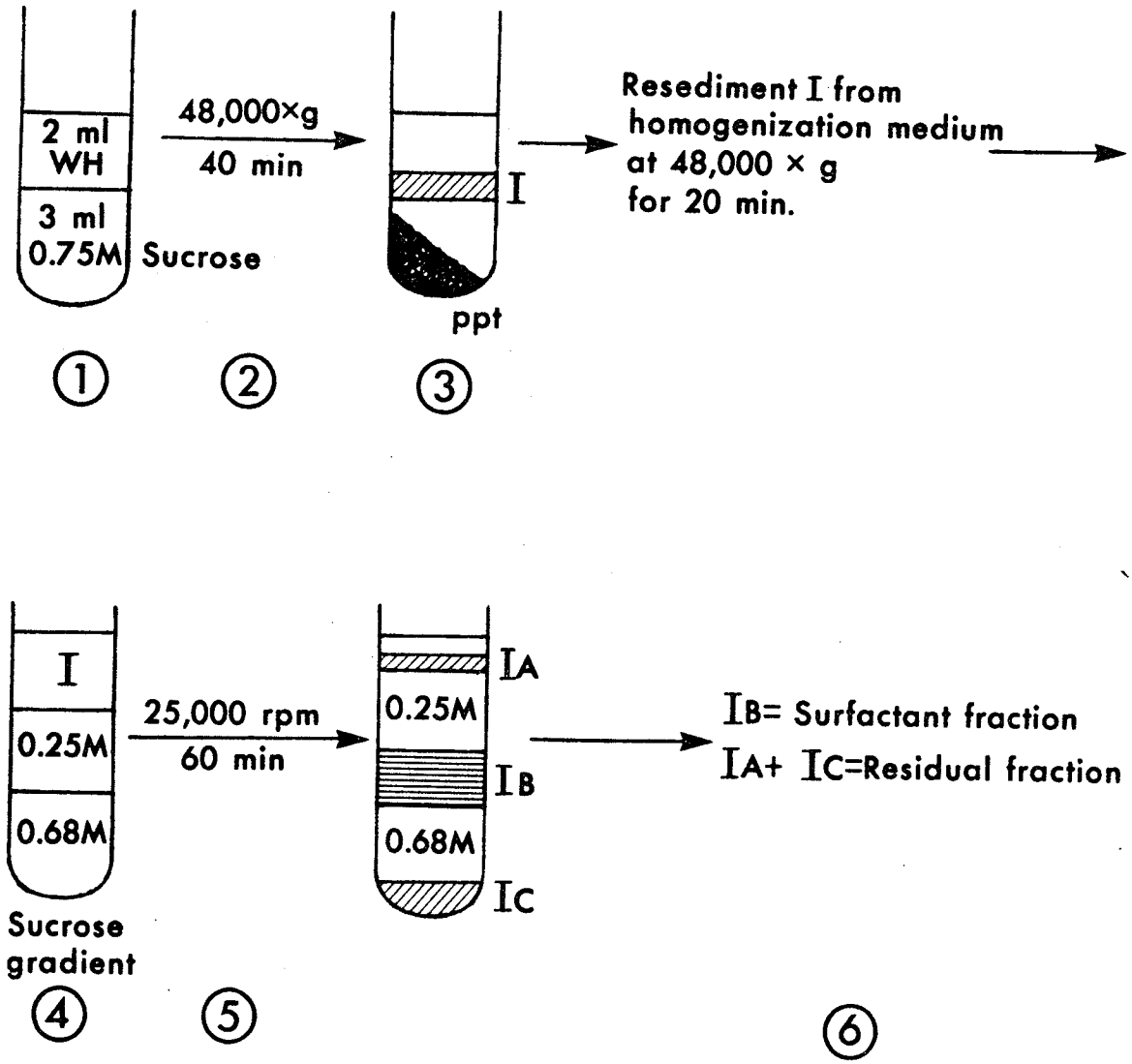


Fig. 4 A flow diagram of the procedures needed to isolate pulmonary surfactant fraction

lower phase was then treated once with chloroform:methanol : water (3:48:47, v/v). The resultant lower phase lipid extract was made to 5 ml with a few drops of methanol.

The phospholipids, except disaturated PC, were separated by thin layer chromatography (Whatman Inc. instructions). The lipid extracts were applied onto 20 x 20 cm Linear-K preadsorbent silica gel TLC plates and allowed to dry thoroughly at room temperature. The plates were first run to the 12 cm mark in a solvent system which contained chloroform: methanol: acetic acid: water (50:25:8:3, v/v) in a developing tank, hot air dried, and then fully developed with chloroform:methanol (9:1, v/v). After TLC separation, the plates were removed and sprayed with 5% sulfuric acid and then charred for 15-20 minutes at 160 C. Phospholipid spots were viewed under ultra-violet light, scraped, and placed into individual tubes.

Disaturated PC was isolated by the method described by Mason et al[164] using osmium tetroxide in carbon tetrachloride (0.1 g/ml). An aliquot of the sample was evaporated to dryness and the residue was redissolved in 0.5 ml of the osmium tetroxide-carbon tetrachloride solution. After 15 minutes, the solution was evaporated and the residue was redissolved in chloroform:methanol (20:1, v/v). This material was then applied to an aluminum oxide column on a glass wool plug in the neck of a Pasteur pipette. The first elution with 10 ml of chloroform:

methanol (20:1, v/v) was discarded. The second elution with 5 ml of chloroform:methanol: 7 M ammonium hydroxide (70:30:2, v/v) was collected in glass tubes.

For the estimation of lipid phosphorus, perchloric acid (72%, w/v) was added to each tube and the mixture was digested at 160°C for two hours. After cooling, 4 ml of water was added, followed by 0.2 ml of 5% ammonium molybdate and 0.2 ml of the reducing agent (15 g sodium bisulfite, 0.5 g sodium sulfite, and 0.25 g of 1-amino-2-naphthol-4-sulfonic acid per 100 ml of deionized H_2O). The mixture was heated in a boiling water bath for 20 minutes, cooled, and centrifuged to sediment the silica gel. Absorbance of the clear supernatnat was measured at 660 nm or 820 nm[165].

7. Estimation of Hydroxyproline

The method for the determination of hydroxyproline was a combination of those described by Madia et al[93] and Stegemann and Stalder[166]. Fresh tissues were extracted with 0.5 M acetic acid (4 ml/g wet weight) in a Potter-Elvehjem homogenizer and the homogenate was left to stir for about 4 hours at 4°C. After removal of the acetic acid-insoluble material by centrifugation, 0.5 ml of the supernatant was made to 1 ml with distilled H_2O. 1 ml of chloramine-T solution was added to the sample, mixed, and

allowed to stand at room temperature for 20 minutes. Then 1 ml of aldehyde/perchloric acid (15 g p-dimethyl-amino-benzaldehyde in 26 ml of 60% perchloric acid and made to 100 ml with n-propanol) was added, mixed thoroughly, and left for 15 minutes in a shaking water bath at 60°C. The samples were cooled and the absorbance read at 550 nm.

8. Assay of Adenylate Cyclase Activity

The enzyme activity was measured by the method of Drummond and Duncan[102] using (8-^{14}C)-ATP as a substrate. The final concentration in the reaction mixture (100 ul) was 40 mM Tris HCl buffer (pH 7.5), 25 mM caffeine, 5.5 mM KCl, 10 mM $MgCl_2$, 20 mM phosphoenolpyruvate, 130 ug/ml of pyruvate kinase, 2 mM cyclic AMP, 0.56 mM ATP containing 10 uCi (8-^{14}C) ATP, and about 100 ug tissue protein. After a 3 minute preincubation at 37°C, the reaction was started with ^{14}C-ATP, allowed to run for 10 minutes, and then stopped by immersing the reaction tubes in boiling water for 3 minutes. The controls were similarly treated except that they were boiled before the addition of ^{14}C-ATP. The reaction mixtures were centrifuged at 2000 rpm for 15 minutes in the cold room. 75 ul of the clear supernatants were 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 containing 4.9 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-(2-(5-phenyl-oxazolyl)) benzene per liter of toluene. The radioactivity was counted in a Searle Mark III counter, and the amount of cyclic AMP was calculated from the specific activity of the ^{14}C-ATP used as substrate. Counts were corrected for radio-activity in the cyclic AMP spot of each boiled sample.

9. Assay of cyclic AMP Phosphodiesterase Activity

The method of Butcher and Sutherland[167] was followed. In a total volume of 0.9 ml, the assay medium contained 40 mM Tris- 40 mM Imidazol buffer (pH 7.5) and 3 mM magnesium acetate, 1.2 mM cyclic AMP, 0.1 ml of 5'-nucleotidase (0.25 U, Sigma grade III) dissolved in 10 mM Tris-HCl containing 0.5 mM magnesium acetate (pH 7.5), 0.167 mM Ca^{2+} whenever present, and 75-100 ug of tissue protein. The reaction was initiated, after pre-incubation for 3 minutes at 30°C, by the addition of cyclic AMP and allowed to continue for 30 minutes, then stopped by adding 0.1 ml 55% ice-cold trichloroacetic acid. The controls were similarly treated except that the trichloro-acetic acid was added before the cyclic AMP. The samples were centrifuged to sediment the denatured proteins. A 0.5 ml aliquot of the clear supernant had added to it 0.5 ml of 55% ammonium molybdate in 1.1 N H_2SO_4, mixed and 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 tubes were allowed to stand for 7 minutes at room temperature and the absorbance was read at 660 nm.

10. Assay of the Ca^{2+}-dependent Regulatory Protein (Calmodulin)

The preparation of the testing sample and the method of assay were as described by Sharma and Wang[137]. The heat-stable, calcium-sensitive protein activator was prepared by incubating an aliquot of the tissue sample (2-5 ml) in a boiling water bath for 2 minutes, then cooled on ice, and centrifuged at 10,000 x g for 5 minutes. The supernatant was used for the assay. The calmodulin was assayed by measuring the stimulation of calmodulin-deficient phosphodiesterase (gift from Dr. Rajendra K. Sharma, Biochemistry Dept., University of Manitoba) in the absence and presence of added calcium (0.17 mM) with or without 0.1 mM EGTA being present. The procedure for the assay was the same as that described for cyclic AMP phosphodiesterase, except that different amounts of the calmodulin preparations with appropriate dilutions were used in place of the regular tissue sample, and calmodulin-deficient phosphodiesterase was included in the assay volume.

11. Assay for Heat-stable Phosphodiesterase inhibitor protein

The method of analysis was a slight modification of the batch DEAE-cellulose procedure described by Wang and Desai (140). An aliquot of the sample was incubated in boiling water bath for 2 minutes, then cooled on ice, and centrifuged at 10,000 x g for 5 minutes. 0.5 ml of the supernatant was adjusted to 0.1 M NaCl and 0.1 mM EGTA with the use of solutions of 2 M Nacl and 10 mM EGTA, respectively. The solution was then mixed with 3 times the sample volume of a DEAE-cellulose slurry containing 50% of DEAE-cellulose (in bed volume) and prepared in a buffer of 20 mM Tris-HCl, 1 mM imidazole, pH 7.5, containing 1 mM magnesium acetate, 0.1 mM EGTA, 15 mM B-mercaptoethanol, and 0.2 M NaCl. The mixture was then stirred gently for a while and centrifuged at 10,000 x g for 5 minutes. The supernatant was used for the assay since the calmodulin becomes bound to the settled DEAE-cellulose. The heat-stable calmodulin becomes bound to the settled DEAE-cellulose. The heat-stable calmodulin-binding protein was assayed by measuring the ability of different concentrations of the sample to inhibit the Ca^{2+} -activable PDE activity when maximally activated in the presence of calmodulin.

12. SDS-polyacrylamide Gel Electrophoresis

The method was that described by Weber and Osborn[168]. The 105,000 x g supernatnat (100 ug protein) was incubated at 37°C for 2 hours in 0.01 M sodium phosphate buffer, pH 7.0, 1% in SDS, 1% in B-mercaptoethanol, 0.015% Bromophenol blue, and 6 M urea. The protein solution was applied to 7.7% SDS-polyacrylamide gel (in gel buffer consisting of 6.8 g NaH_2PO_4, 20.45 g Na_2HPO_4, and 2 g of SDS per litre) in 10 cm glass tubes. The two compartments of the electrophoresis apparatus were filled with the gel buffer diluted 1:1 with distilled water, and the electrophoresis was carried out for 4½ hours at a constant current of 8 ma per gel with the positive electrode in the lower chamber. After electrophoresis, the gels were removed and stained in Coomassie brilliant blue for 10 hours, and then destained in 7.5% acetic acid and 5% methanol. The relative mobility of the proteins was calculated as follows:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}} \times \frac{\text{length of gel before staining}}{\text{length of gel after staining}}.$$

13. Electron Microscopy

For each animal, tissue samples were taken immediately from the peripheral areas of the lung, cut into pieces smaller than 1 mm cube and then immersed in Millonig's buffered glutaraldehyde for 30 minutes. The tissue slices were washed three times

in Millonig's buffer and then fixed in Millonig's buffered OsO_4 for 1½ hours. After fixation, the tissue slices were put in 2% Uranyl acetate for 25 minutes and then dehydrated in a graded series of alcohol, followed subsequently in a 1:1 and then a 1:3 mixture of absolute alcohol and Spurr plastic (at 1½ hours each time), finally embedded in 100% Spurr plastic for ½ hour in small gelatin capsules that have been dried in the oven. The capsules were left overnight in 65-70^{o}C oven to harden. After the capsules with plastic and tissue slices were sufficiently hard, the gelatin capsules were soaked off the blocks with warm water. Excess plastic was trimmed off the end of each block to the shape of a shallow pyramid. Thin sections of tissues were cut and impregnated with lead citrate prior to viewing(169).

E. RESULTS

In order to assess the severity of experimental diabetes induced in the streptozotocin-treated animals, body weights as well as serum and urine glucose levels were measured (Table I). Eleven days following treatment with streptozotocin, the diabetic rats experienced an average weight gain of 29 g, whereas the control and the insulin-treated diabetic rats gained average weights of 73 g and 83 g, respectively. In addition, the diabetic animals were clinically diabetic with positive urinary glucose. The elevated serum glucose in the diabetic rats further confirmed the difference between streptozotocin-treated and control rats.

Despite the differences in body weights of the control and diabetic rats, the lung weights relative to body weights were unchanged (Table II). However, the diabetic condition caused a significant decrease in absolute lung weight and had a marked effect on lung constituents of the animals. Lung protein content of the diabetic animals was not significantly different from that of the control groups, but the DNA concentration was significantly lower when compared as mg DNA/g lung tissue. However, the protein/DNA ratio showed a marked increase in the diabetic lung. This increase was reversed to control value in the insulin-treated diabetic rats. The

TABLE I

BODY WEIGHTS, SERUM AND URINE GLUCOSE LEVELS OF CONTROL, DIABETIC AND INSULIN-TREATED DIABETIC RATS

Values are mean ± SEM of three independent experiments performed with 10 animals in each experimental group.

MEASUREMENT	CONTROL	DIABETIC	INSULIN-TREATED DIABETIC
Body Weight (g)			
Basal (day 0)	119 ± 9	122 ± 5	120 ± 3
At time of sacrifice (day 11)	192 ± 7	151 ± 5	203 ± 7
Change in Body Weight	73 ± 8	29 ± 1	83 ± 6
Serum Glucose (mg/100 ml) (on day 11)	105 ± 2	423 ± 18	122 ± 4
Urine Glucose (day 4-11)	0	3+	0

TABLE II

EFFECT OF DIABETES ON RAT LUNG ALVEOLAR TISSUE COMPOSITION

Values are mean ± SEM of three independent experiments performed with 10 animals in each experimental group.

MEASUREMENT	CONTROL	DIABETIC	INSULIN-TREATED DIABETIC
Lung Weight (g)	1.08 ± .19	0.086 ± .10	1.01 ± .09
g/100 g Body Weight	0.56 ± .02	0.50 ± .02	0.50 ± .05
Protein (mg/g Lung Tissue)	161 ± 3	150 ± 2	164 ± 4
DNA (mg/g Lung Tissue)	5.67 ± .34	3.80 ± .23	6.17 ± .06
Protein/DNA (mg/mg)	28.2 ± .5	39.5 ± .4	26.6 ± 1.5
Glycogen (mg/g Lung Tissue)	11.01 ± .51	7.36 ± .22	-
Hydroxyproline (µg/g Lung Tissue)	13.70 ± .39	21.25 ± .42	-
Phospholipid (mg/g Lung Tissue)	17.62 ± .23	13.72 ± .26	-

hydroxyproline content of acetic acid extracts of the lung tissue was estimated as an index of the collagen content of the tissue. The diabetic lung tissue showed a higher hydroxyproline content than the control. The lung glycogen content showed a significant reduction in the diabetic animals.

As shown in Tables II and III, there was a marked reduction in the phospholipid content of lung tissues of the diabetic animals when compared to the controls. A similar difference in phospholipid between diabetic and control animals appeared significantly in the surfactant fractions of the lung tissues; the residual fractions did not change appreciably. Interestingly, the percent of phosphatidylcholine in the surfactant fractions of the diabetic lung was only slightly changed. However, the percents of disaturated phosphatidylcholine and of lyso-phosphatidylcholine were significantly decreased in the diabetic lungs. Also there was a 2-3 fold decrease in the percent of phosphatidylglycerol in the surfactant fractions of the diabetic lungs when compared to control values.

Since pulmonary synthesis of proteins and surfactant are generally associated with the alveolar Type II cells, ultrastructural studies were undertaken in order to assess the effect of streptozotocin-induced diabetes on this lung cell type. As shown in Figures 5-7, the most obvious alterations in the ultrastructure of the Type II cells after eleven

TABLE III

INFLUENCE OF DIABETES ON RAT LUNG ALVEOLAR TISSUE PHOSPHOLIPIDS

Values are mean ± SEM of three separate experiments performed in triplicate samples.

PHOSPHOLIPIDS	CONTROL	DIABETIC
Residual Fraction * (mg/g Lung Tissue)	2.94 ± .13	1.98 ± .18
Surfactant Fraction* (mg/g Lung Tissue)	14.19 ± .22	11.66 ± .27
Phosphatidylcholine (% of Fraction)	65.80 ± .12	60.71 ± .13
Disaturated Phosphatidylcholine (% of Phosphatidylcholine)	39.01 ± .16	24.29 ± .10
Lyso Phosphatidylcholine (% of Phosphatidylcholine)	1.50 ± .17	0.69 ± .19
Phosphatidylglycerol (% of Fraction)	6.53 ± .19	2.25 ± .13

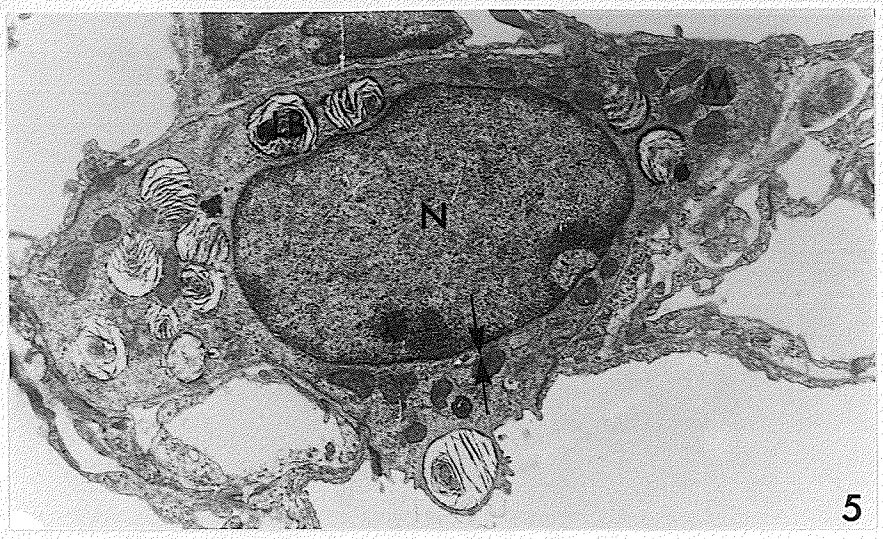
*See Experimental Procedures for definition of residual and surfactant fractions.

days following injection of streptozotocin were in the endoplasmic reticulum. Some of the cisternae were dilated and the contents were less dense than the surrounding cytoplasm. The degree of dilation varied between massive expansions and localized dilations adjacent to normal granular endoplasmic reticulum. The majority of the enclosing membranes were visible and lined by ribosomes but on the expansions, long areas of membrane were observed to be free of ribosomes. Other alterations of the Type II cells included disintegration of the mitochondrial cristae and dilation of saccules in the golgi complex. There were no changes in the number per cell and the size of the lamellar bodies; however, these were not quantitated.

Potential impairment of the adenylate cyclase activity of lung alveolar tissue was investigated in tissue homogenates and particulate fractions of control and streptozotocin-diabetic rats (Table IV). The basal adenylate cyclase activity was decreased in the particulate fractions of diabetic rat lung tissues. Insulin treatment of the diabetic rats showed a restoration of the enzyme activity to control values. The specific and total activities of adenylate cyclase in the diabetic tissue homogenates were markedly higher than in the control samples; these activities were restored to control values by insulin treatment. Of interest was the finding that the tissue homogenates of each experimental group pre-

FIG. 5 Characteristic features of the lung alveolar Type II cell from a saline - injected control rat illustrating a central nucleus(N), lamellar bodies(LB) of varying sizes, mitochondria(M), and a narrow profile of granular endoplasmic reticulum(arrowheads). x 10,300.

FIG. 6 General appearance of the lung alveolar Type II cell from a streptozotocin - treated rat killed 11 days after injection. In comparison with Fig. 5, there is focal dilation of granular endoplasmic reticulum(dGER). Undilated granular endoplasmic reticulum(arrowhead) and a dilated Golgi saccule(G) are also present. There is disintegration of the mitochondrial cristae(dM). The nucleus(N) and lamellar bodies(LB) appear unaltered. x 15,400.


N
M
5

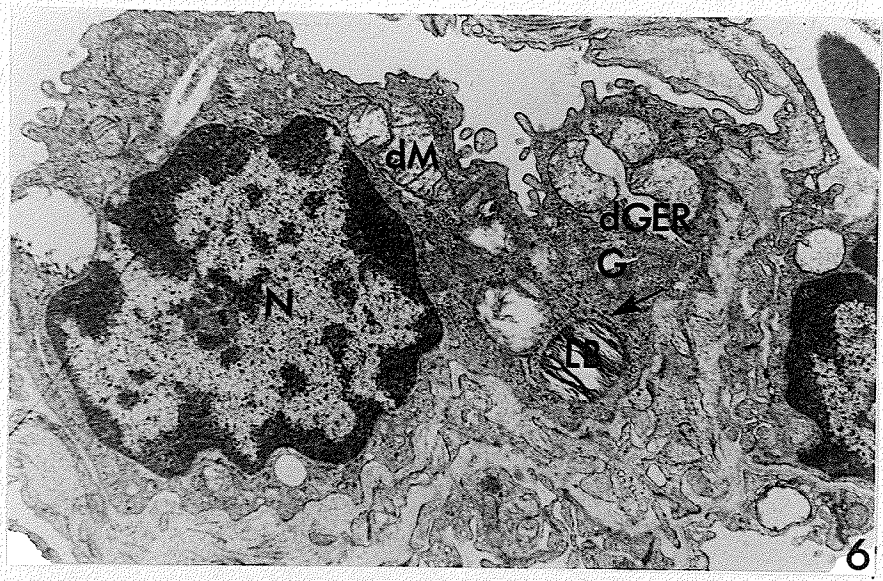

dM
dGER
G
N
6

FIG. 7 Massively dilated granular endoplasmic reticulum(dGER) in a lung alveolar Type II cell from a streptozotocin - treated rat, with the membrane free of ribosomes. A narrow profile of granular endoplasmic reticulum showing loss of ribosomes(arrowhead) and a dilated Golgi saccule(G) are also present. There is disorganization of the internal structure of the mitochondria(dM). The lamellar bodies(LB) and multivesicular body(MVB) appear unchanged. x 35,100.

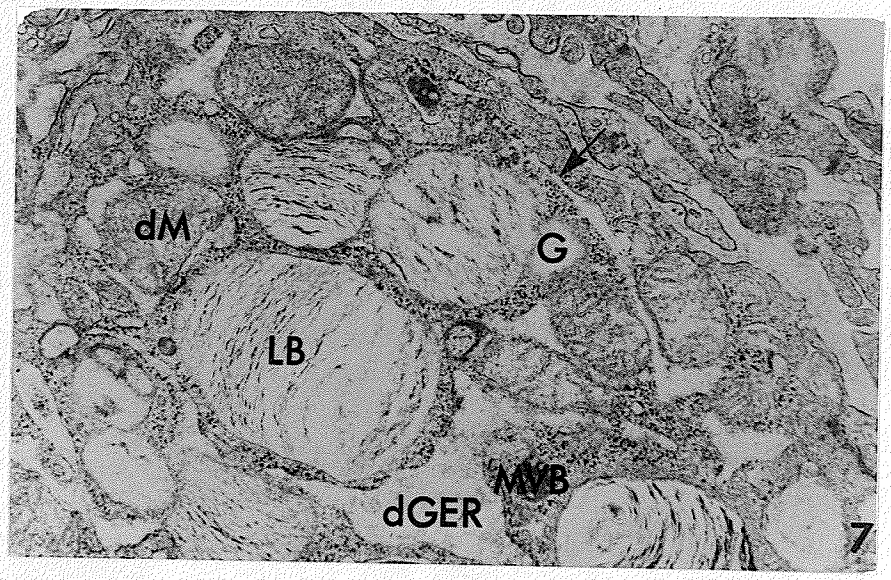

dM
G
LB
MVB
dGER
7

TABLE IV

EFFECT OF DIABETES ON ADENYLATE CYCLASE
ACTIVITY OF RAT LUNG ALVEOLAR TISSUE

Values represent mean ± SEM of three independent experiments performed in triplicate samples.

SAMPLES	CONTROL	DIABETIC	INSULIN-TREATED DIABETIC
Whole Homogenate			
Specific activity (pmole cAMP/min/mg protein)	226 ± 13	386 ± 11	216 ± 9
Total activity (nmole cAMP/min/g tissue)	36 ± 5	58 ± 5	35 ± 7
Particulate Fraction			
Basal activity (pmole cAMP/min/mg protein)	197 ± 8	125 ± 10	192 ± 12

sent higher specific adenylate cyclase activities when compared to the basal activities in the particulate fractions.

In order to assess the physiological significance of the cytoplasmic factor(s) in the regulation of rat lung adenylate cyclase, the effects of different concentration of the supernatnat fractions prepared from control, diabetic and insulin-treated diabetic rat lungs on basal adenylate cyclase activity in the particulate fractions were examined. Figure 8 shows that in diabetic rat lungs the supernatant fraction activated the particulate adenylate cyclase activity to a much higher extent than in the control lungs. As shown in Figure 9, the activating capacity of the diabetic supernatant fraction remained higher and to the same extent when examined in the presence of the control particulate fraction. Since the time of appearance of the activity of the cytoplasmic factor(s) has been observed by Nijjar[121] to coincide with the time of cessation of rapid alveolar multiplication in the rat lungs (23 days postpartum), the effect of age of the rat at the time of induction of diabetes on the activation of particulate adenylate cyclase by supernatant fractions was examined. Figure 10 shows that induction of streptozotocin-diabetes to rats 14 days postpartum caused a 2.50-fold increase in the activity of the cytoplasmic factor(s), whereas rats rendered diabetic at 39 days postpartum showed a 1.82-fold increase in activity.

FIG. 8 Effects of different concentrations of the supernatant fractions prepared from Control(●), Diabetic(Δ), and Insulin-treated Diabetic(■) rat lung alveolar tissues on activation of basal adenylate cyclase activity of their respective particulate fractions. Net activation of Adenylate cyclase activity = Increased activity - basal activity. Each point is the mean value of three independent experiments assayed in triplicate samples.

Fig. 8

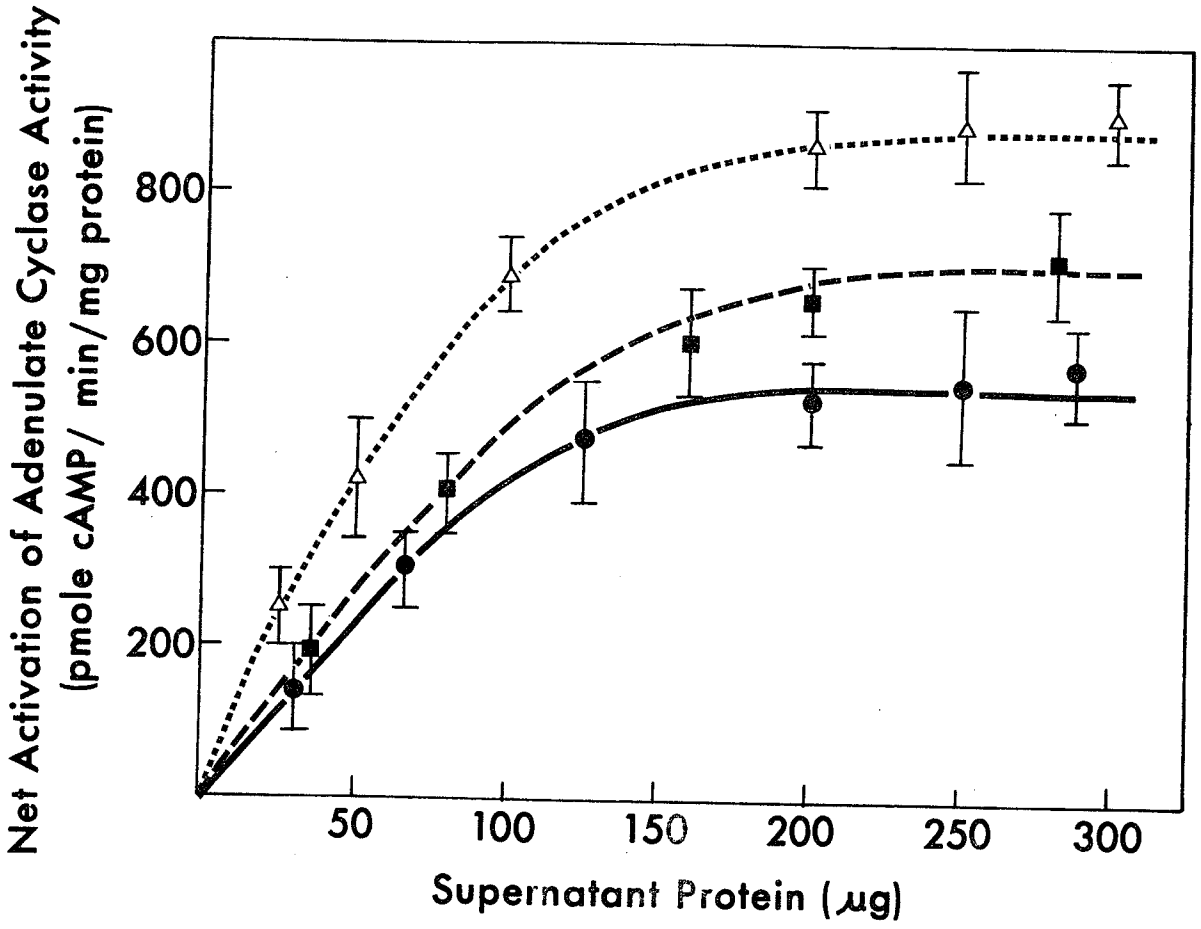

Net Activation of Adenulate Cyclase Activity
(pmole cAMP/ min/mg protein)
800
600
400
200
50
100
150
200
250
300
Supernatant Protein (μg)

FIG. 9 Activation of basal adenylate cyclase activity in the control particulate fraction by different concentrations of the supernatant fractions prepared from Control(●), Diabetic(▲), and Insulin-treated Diabetic(■) rat lut alveolar tissues. Net activation of Adenylate cyclase activity = Increased activity - basal activity. Each point represents the mean value of three separate experiments performed in triplicate samples.

Fig. 9

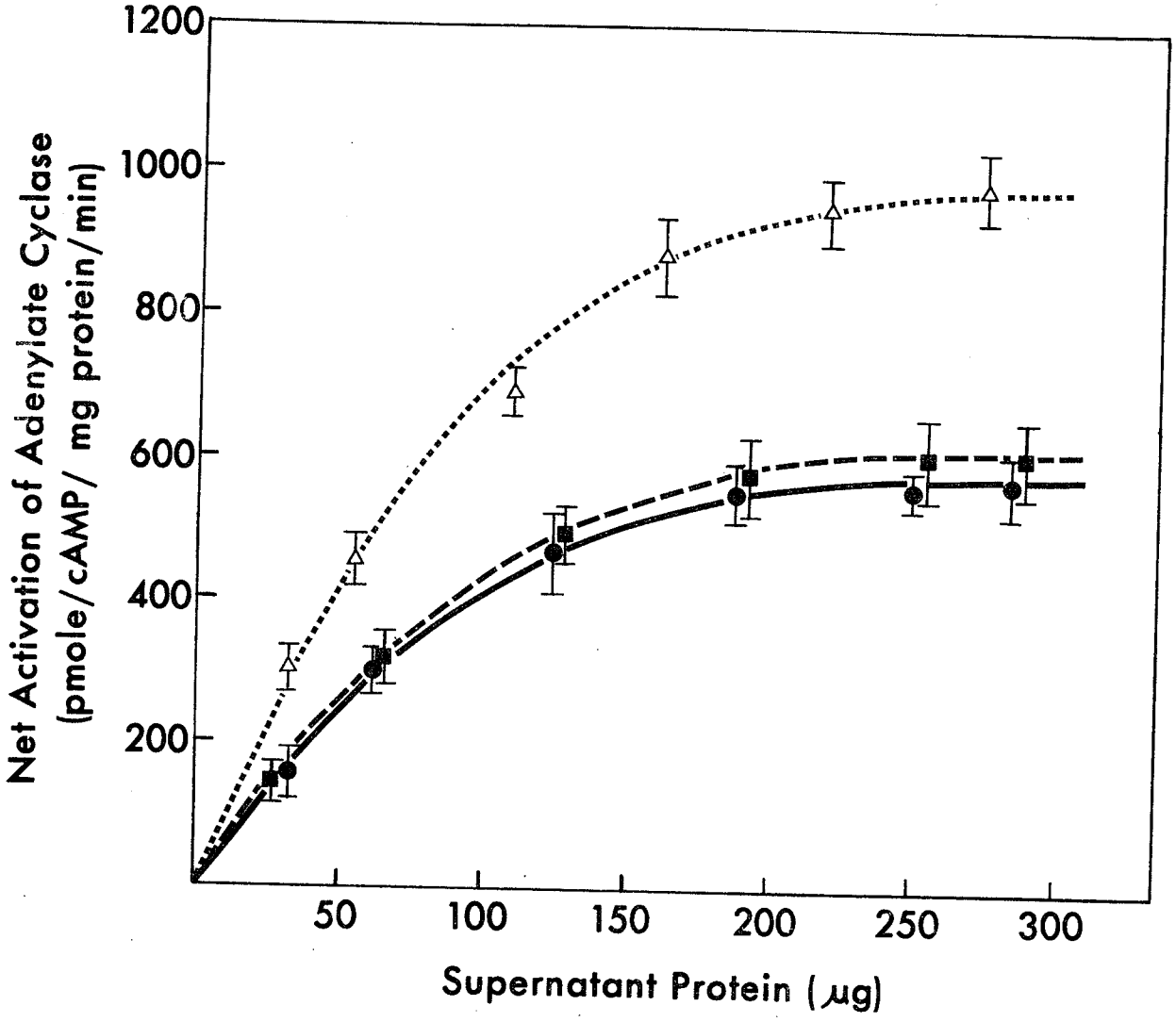
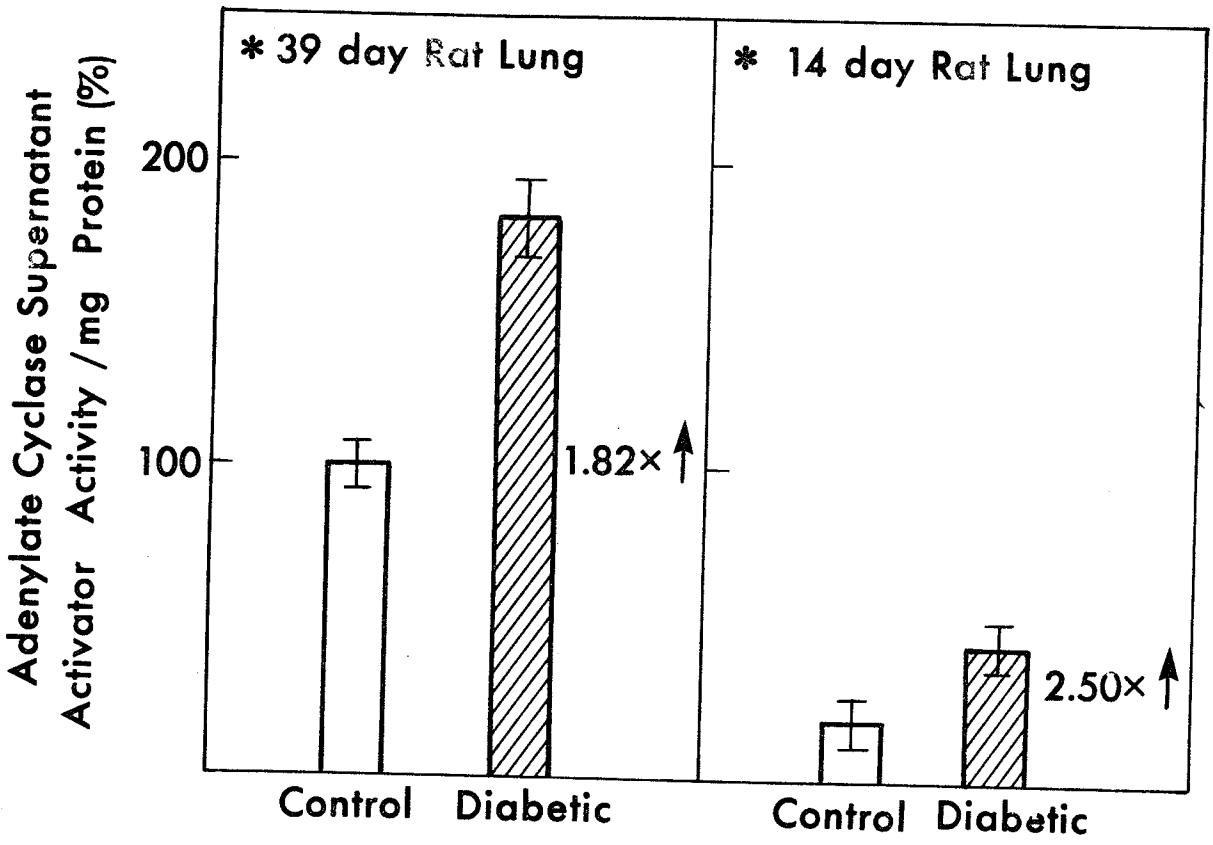

1200
1000
800
600
400
200
Net Activation of Adenylate Cyclase
(pmole/cAMP/ mg protein/min)
50
100
150
200
250
300
Supernatant Protein (μg)

FIG. 10 Effect of age of rat at time of induction of diabetes(*) on the activation of particulate adenylate cyclase by supernatant fractions. The values represent mean values from three independent experiments. 39 day old control animal values were set at 100% ± SEM.

Fig. 10


* 39 day Rat Lung
* 14 day Rat Lung
Adenylate Cyclase Supernatant
Activator Activity /mg Protein (%)
200
100
1.82×
2.50×
Control
Diabetic
Control
Diabetic

The possibility has been raised that the lung cytoplasmic factor(s) may be calmodulin or calmodulin bound to another protein. It has therefore become necessary to examine such a possibility in this study. As shown in Table V, calmodulin activity was demonstrated in the rat lung tissue. The activity was distributed amongst the lung subcellular fractions. Of particular interests were the demonstration of calmodulin activity in the particulate and supernatant fractions, and the 2-3-fold higher activity in the supernatant fraction when compared to the activity in the particulate fraction. In seeking a significance for the calmodulin activity in the particulate fraction, the possibility was considered that Ca^{2+} and calmodulin may regulate the basal adenylate cyclase activity of the particulate fraction. In the first group of experiments, the addition of 100 uM Ca^{2+} to the assay system had an inhibitory effect on the particulate adenylate cyclase activity (Table VI). A similar effect was demonstrated with 15 ug of calmodulin. Removal of Ca^{2+} from the system by the addition of 100 uM EGTA showed a slight but significant stimulatory effect on the adenylate cyclase activity. The addition of 100 uM Ca^{2+} and 15 ug calmodulin to the assay system caused no significant change in the particulate adenylate cyclase activity. Because of the possibility that the observed inhibitory effects of Ca^{2+} and of calmodulin may be due to endogeneous Ca^{2+} and calmodulin, the second group of experiments was performed with

TABLE V

CALMODULIN ACTIVITY IN RAT LUNG ALVEOLAR TISSUE HOMOGENATE AND SUBCELLULAR FRACTIONS

Values represent mean ± SEM of 3 independent experiments performed in triplicate samples.

	CALMODULIN ACTIVITY (pmole Pi/min/mg protein)
Whole Homogenate	378 ± 7
15,000 x g supernatant	284 ± 10
105,000 x g pellet (particulate fraction)	82 ± 13
105,000 x g supernatant (supernatant fraction)	189 ± 4

For the assay of calmodulin, an aliquot of the sample was boiled for 2 minutes, centrifuged and the supernatant was analyzed for ability to activate a standard amount of calmodulin-deficient PDE in the presence of 100 μM Ca^{2+}. In the assay system, the PDE reaction was coupled to a 5'nucleotidase reaction, hence calmodulin activity is expressed as pmole Pi released/min/mg protein.

TABLE VI

EFFECTS OF CALCIUM AND CALMODULIN ON ADENYLATE CYCLASE ACTIVITY OF RAT LUNG ALVEOLAR TISSUE PARTICULATE FRACTIONS
(expressed as pmole cAMP/min/mg protein)

Values are mean ± SEM of two independent experiments performed in triplicates.

	ADENYLATE CYCLASE ACTIVITY
Undialysed Particulate Fraction	
Basal	225 ± 11
+ Ca^{2+} (100 µM)	182 ± 2
+ Calmodulin (15 µg)	189 ± 5
+ EGTA (100 µM)	255 ± 7
+ Ca^{2+} (100 µM) + Calmodulin (15 µg)	240 ± 4
*Dialysed Particulate Fraction	
Basal	175 ± 5
+ Ca^{2+} (50 µM)	176 ± 8
+ Ca^{2+} (100 µM)	189 ± 3
+ Ca^{2+} (100 µM) + Calmodulin (15 µg)	359 ± 10

*The particulate fraction samples were dialysed overnight against 250 ml (two changes) of 100 µM EGTA in 10 mM Tris-HCl, pH 7.4

particulate fractions dialyzed overnight against two volumes of 250 ml of 100 uM EGTA in 10 mM Tris-HCl buffer, pH 7.4. The results are presented in Table VI. Basal adenylate cyclase activity in the dialyzed particulate fraction was lower than that in the undialyzed fraction. Addition of 50 uM Ca^{2+} did not affect the activity. The addition of 100 uM Ca^{2+} caused a small but nonsignificant increase in activity. However, when 100 uM Ca^{2+} and 15 ug calmodulin were added to the dialyzed particulate fraction, there was a marked stimulatory effect on the basal adenylate cyclase. Figure 11 shows that the activation took place in a dose-dependent manner; the inhibitory effect of calmodulin-binding protein on maximal Ca^{2+}-calmodulin activated adenylate cyclase was also demonstrated. The possibility that the activation of particulate adenylate cyclase by the supernatant fraction of the rat lung may be due to the presence of calmodulin in the supernatant fraction was also examined. As shown in Figure 12, different concentrations of EGTA and calmodulin-binding protein in the presence of Ca^{2+} did not affect the supernatant activation of the particulate adenylate cyclase activity.

The cytoplasmic factor(s) responsible for the supernatant activation of lung particulate adenylate cyclase has now been isolated and purified (Nijjar, privileged communication). They are dsignated as Peak I and Peak II according to their elution from a DEAE-cellulose column chromatography.

FIG. 11 Effects of Calmodulin on particulate adenylate cyclase activity and of Calmodulin-Binding Protein on calmodulin-activated particulate adenylate cyclase activity. The particulate samples were previously dialyzed overnight against two volumes of 250ml each time of 100uM EGTA in 10mM Tris-HCl buffer, pH 7.4. All assays were performed at different concentrations of Calmodulin and Calmodulin-Binding Protein in the presence of 100uM Ca^{2+}. Each point represents mean values of four different experiments performed in triplicate samples.

Fig. 11

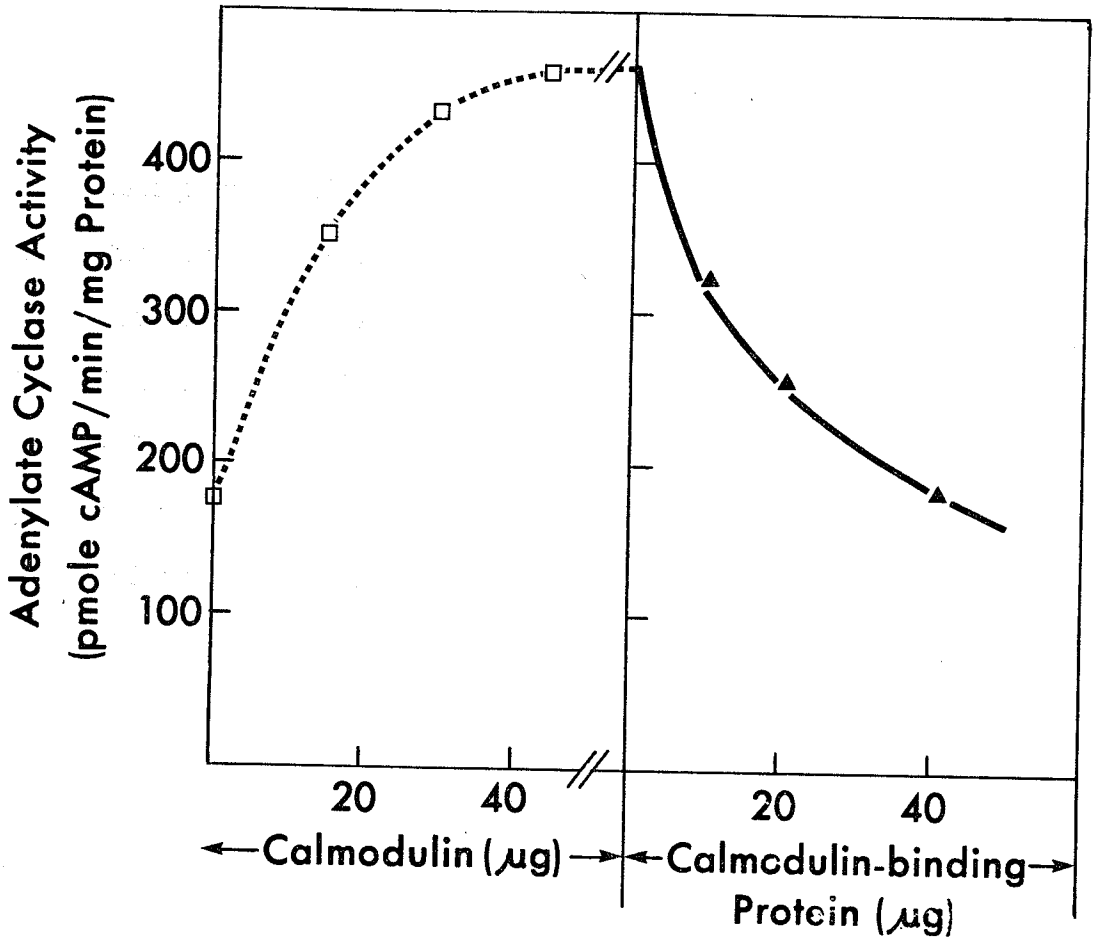
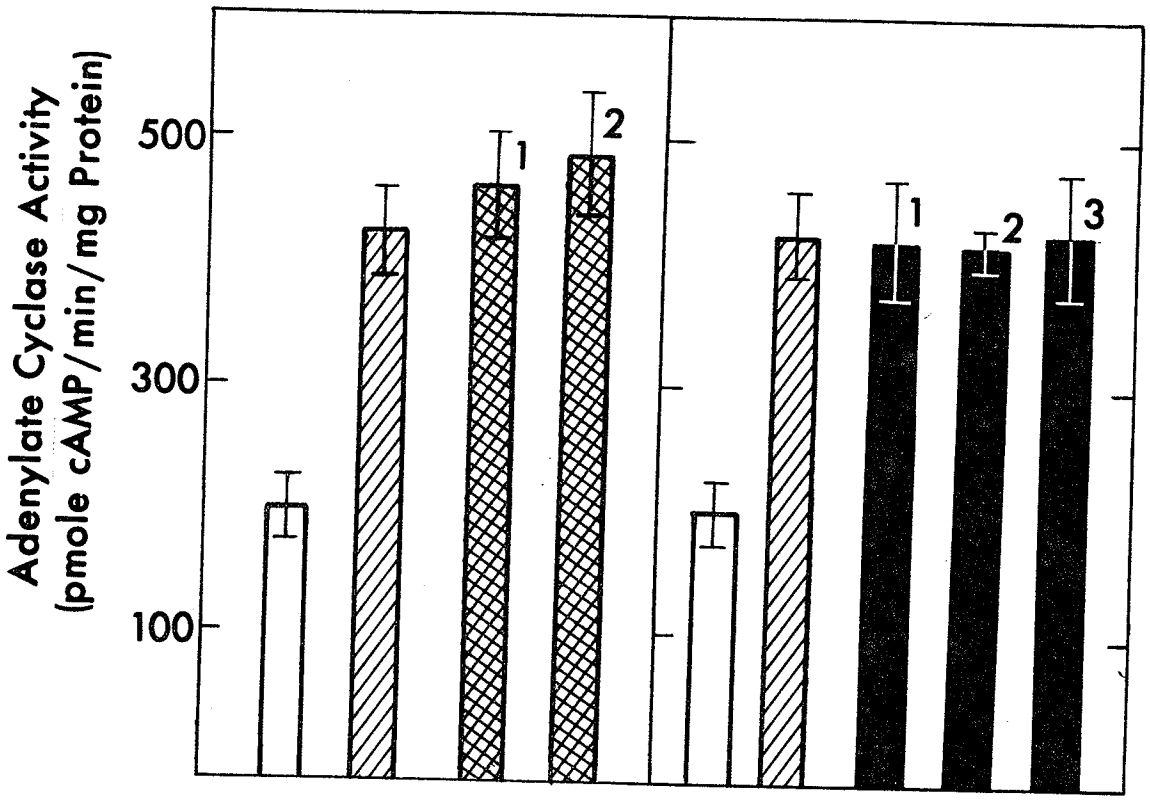

Adenylate Cyclase Activity
(pmole cAMP/min/mg Protein)
400
300
200
100
20
40
20
40
←Calmodulin (μg)→
←Calmodulin-binding→
Protein (μg)

FIG. 12 Effects of EGTA and Calmodulin-Binding Protein on supernatant activation of particulate adenylate cyclase activity. The same protein concentration of particulate and supernatant fractions were used in the assays. Values represent the mean of two independent experiments performed in triplicates.

Fig. 12



Particulate

Particulate + Supernatant

Particulate + Supernatant + EGTA
1. 100 μM
2. 200 μM

Particulate + Supernatant + Ca^{2+} (100 μM)
+ Calmodulin Binding Protein
1. 20 μg
2. 40 μg
3. 80 μg

Peak I comprises of 14,000-15,000 and 65,000 dalton proteins, whereas Peak II is purely of the 65,000 dalton protein. In order to assess the individual roles of the two proteins in the diabetic state, SDS-polyacrylamide gel electrophoretic separation of these proteins from other proteins of the supernatant fractions of control, diabetic and insulin-treated diabetic rat lungs were undertaken (Figure 13). The amounts of the activator proteins were then measured and each expressed as percent of total cytoplasmic proteins. As shown in Table VII, the amount of the low molecular weight protein was unchanged, whereas there was about a 2 fold increase in the amount of the high molecular weight protein in the diabetic lung total cytoplasmic protein. The amount of the high molecular weight protein in the insulin-treated diabetic rat lungs was not different from that in the control samples.

Since insulin receptors have been demonstrated in membrane preparations from normal rat lungs[8] and, in liver tissue, insulin stimulates a membrane-bound cyclic AMP-phosphodiesterase[127,148], it was necessary to examine the effect of diabetes on cyclic AMP-phosphodiesterase (PDE) activity of the rat lung alveolar tissue. As shown in Table VIII, there was no difference in the nature of cyclic AMP-phosphodiesterase present in the particulate and supernatant fractions; both have Ca^{2+}-independent and Ca^{2+}-dependent activities. Data presented here show that there was a depressed cyclic AMP-phosphodies-

FIG. 13 SDS - polyacrylamide gel electrophoresis of 105,000 x g supernatant proteins from Control, Diabetic and Insulin - treated Diabetic rat lung alveolar tissues. Peak I and II represent the cytoplasmic protein fractions (as specified by Nijjar) responsible for the supernatant activation of the rat lung particulate adenylate cyclase enzyme. They are designated as such according to their elution from a DEAE - cellulose column chromatography. Peak I comprise of 14,000 - 15,000 dalton and 65,000 dalton proteins, whereas Peak II is composed of only the 65,000 dalton protein.

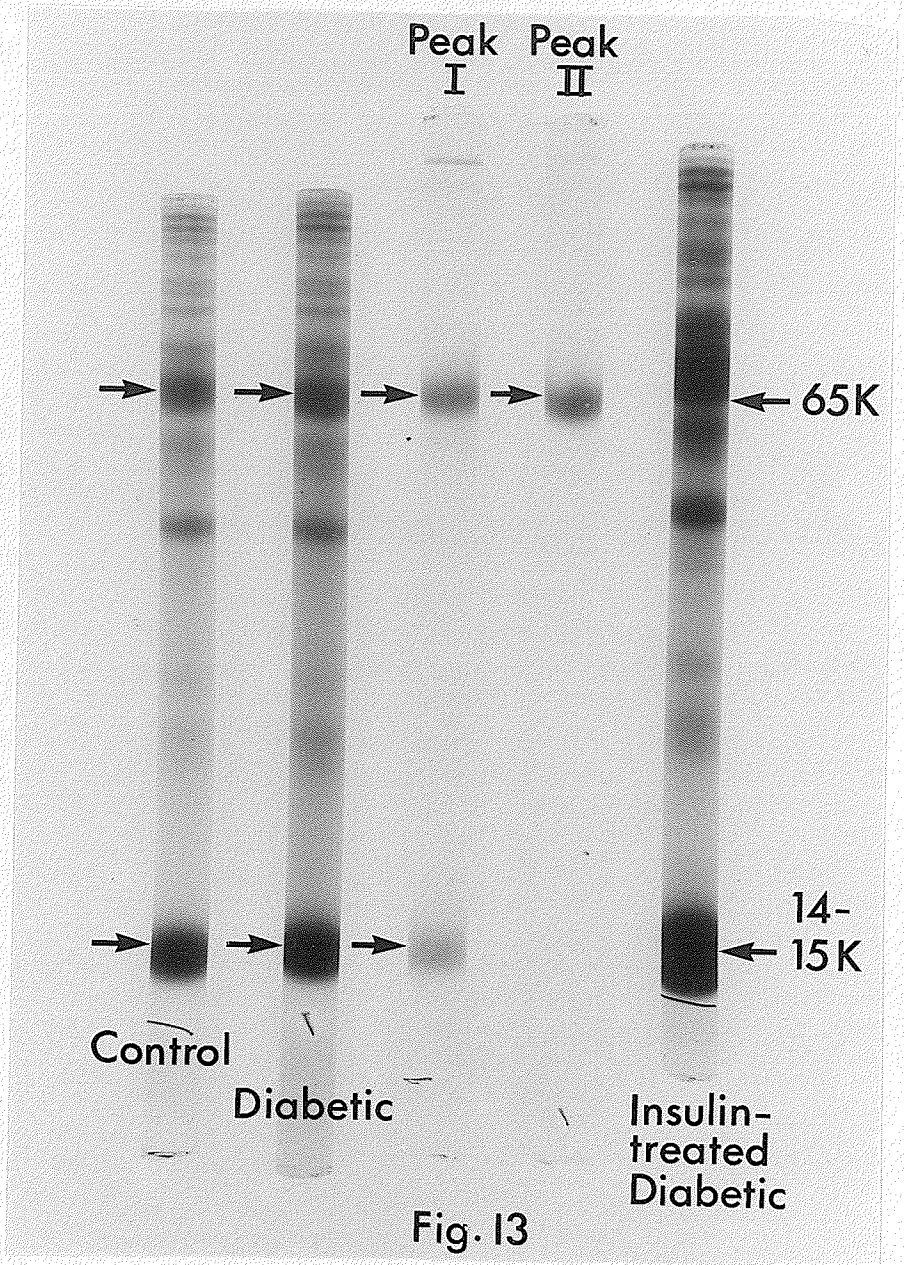

Peak I
Peak II
65K
14-15K
Control
Diabetic
Insulin-treated Diabetic

Fig. 13

TABLE VII

EFFECT OF DIABETES ON RAT LUNG CYTOPLASMIC ACTIVATOR PROTEINS OF ADENYLATE CYCLASE

Values represent mean ± SEM of two independent experiments performed in triplicate samples.

ACTIVATOR PROTEINS	PERCENT OF TOTAL PROTEIN*		
	CONTROL	DIABETIC	INSULIN-TREATED DIABETIC
Low Molecular Wt. (14,000 - 15,000 daltons)	58.8 ± 1.6	59.1 ± 1.1	63.9 ± 2.5
High Molecular Wt. (65,000 daltons)	14.6 ± 1.2	25.2 ± 0.6	15.6 ± 1.0

*The percent of total protein was calculated from the area under each peak obtained from densitometric scanning (SP 8 - 100 UV Pye Unicam Spectrophotometer) of the SDS-polyacrylamide gel electrophoretic separation of the proteins of the 105,000 x g supernatant fraction of rat lung alveolar tissue.

TABLE VIII

EFFECT OF DIABETES ON CYCLIC AMP-PHOSPHODIESTERASE ACTIVITY OF RAT LUNG ALVEOLAR TISSUES

Activities are expressed as nmole Pi released/min/mg protein.
Values are mean ± SEM of three separate experiments performed in triplicates.

FRACTIONS	CONTROL		DIABETIC	
	+ EGTA	+ Ca^{2+}	+ EGTA	+ Ca^{2+}
Whole Homogenate	8.67 ± 1.70	18.50 ± 1.77	5.52 ± 0.54	13.24 ± 1.51
Particulate Fraction*	2.35 ± .35	3.15 ± .07	1.03 ± .06	1.86 ± .40
Supernatant Fraction*	4.90 ± .06	14.00 ± .21	4.40 ± .05	11.61 ± .25

*The lung tissue homogenate was centrifuged at 105,000 x g for 60 minutes. The resulting pellet was used here as the particulate fraction and the supernatant as the supernatant fraction. Cyclic AMP-PDE activity was determined in the absence and presence of added calcium (0.167 mM) with or without 0.1 mM EGTA being present.

terase activity in the diabetic tissue. The particulate PDE activities were depressed about 2 fold compared to the control. However, in the supernatant fraction, the Ca^{2+}-independent PDE activity was unaffected whereas the Ca^{2+}-dependent activity decreased. The possibility that alterations of calmodulin activity may contribute to the depression of the lung tissue PDE in diabetes was examined. Figures 14 and 15 show that in diabetes calmodulin activity was reduced in the particulate fraction but increased in the supernatant fraction. However, as shown in Figure 16, calmodulin activity in the 15,000 x g boiled supernatant from control and diabetic lung tissues was markedly different, about 60% lower in the diabetic when expressed per mg of the lung tissue protein and as total calmodulin activity. Since a heat-stable inhibitor of PDE has been identified in brain tissue[141], the lung tissues were examined for this inhibitory protein. The data presented in Figure 17 show that an inhibitory protein of Ca^{2+}-activatable PDE activity was present in the rat lung and that the activity of this protein was increased in diabetes.

Since adenylate cyclase produces cyclic AMP and cyclic AMP phosphodiesterase hydrolyzes it, a ratio of the activities of these enzymes may give an indication of the cyclic AMP level at any particular moment in the rat lung tissue. On this basis, the data presented in Table IX show that Adenylate cyclase/cyclic AMP-PDE ratio was elevated during diabetes.

FIG. 14 Determination of Calmodulin activity in the supernatant fractions from Control(●), Diabetic(Δ), and Insulin-treated Diabetic(■) rat lung alveolar tissues. The Calmodulin activity was assayed by the ability of the boiled samples to stimulate Calmodulin-deficient PDE. See Experimental Procedures for details. Values represent mean of three independent experiments performed in triplicate samples.

Fig. 14

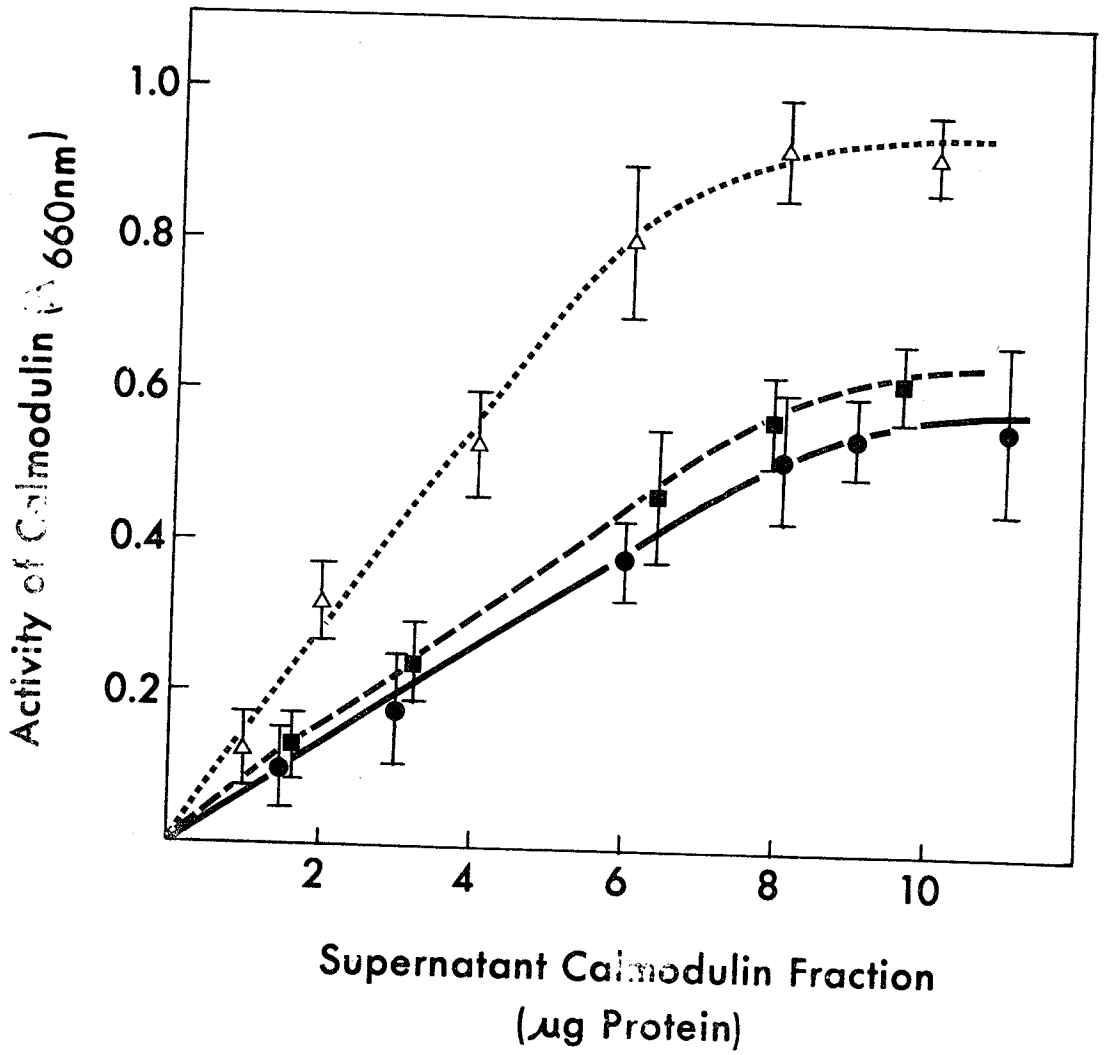

1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
Supernatant Calmodulin Fraction
(μg Protein)

FIG. 15 Determination of Calmodulin activity in the particulate fractions from Control(●), Diabetic(Δ), and Insulin-treated Diabetic(■) rat lung alveolar tissues. Calmodulin activity was assayed by measuring the stimulation of Calmodulin-deficient PDE as described in Experimental Procedures. Each point is the mean value of three independent experiments performed in triplicate samples.

Fig. 15

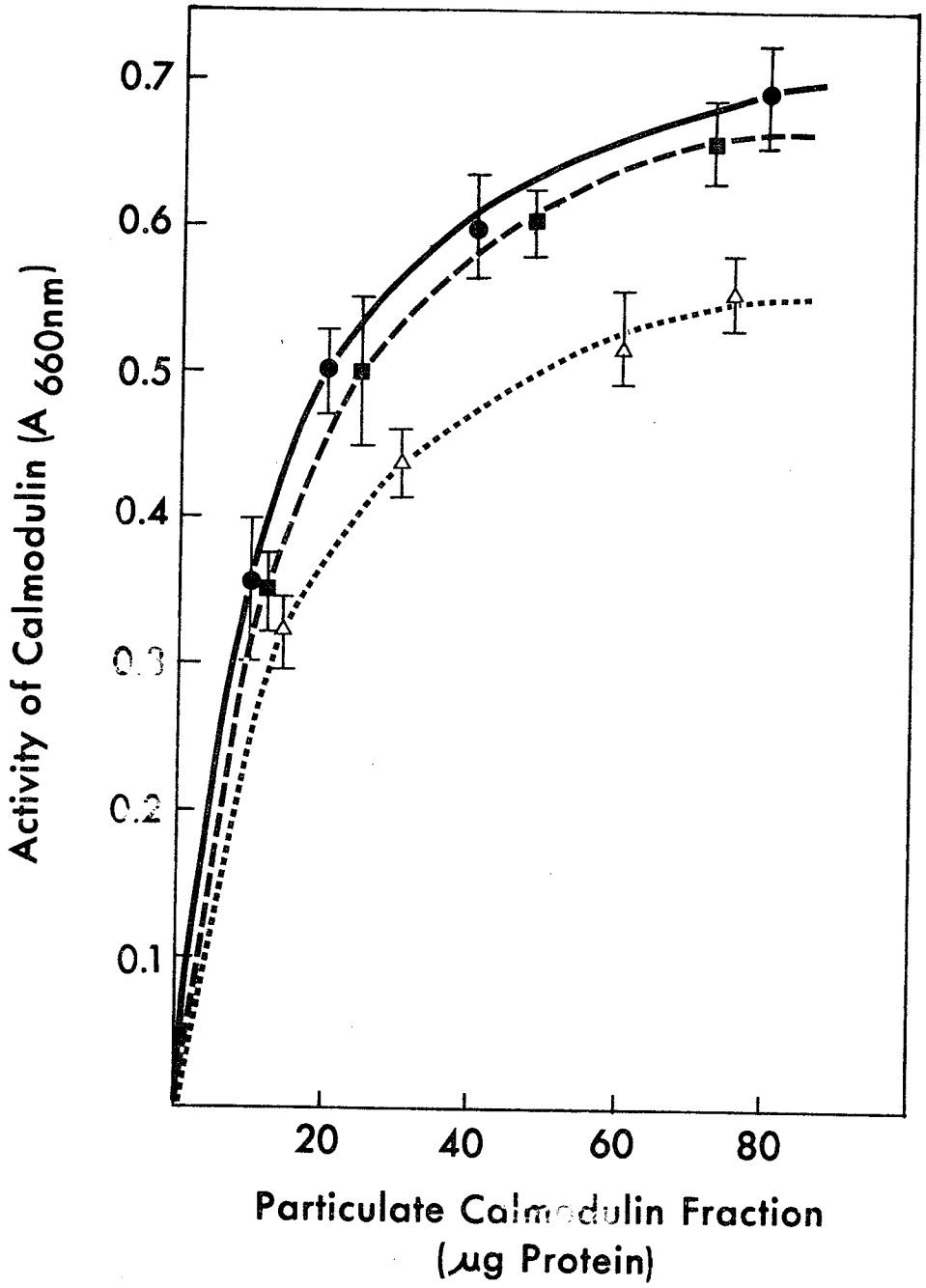

0.7
0.6
0.5
0.4
0.3
0.2
0.1
Activity of Calmodulin (A 660nm)
20
40
60
80
Particulate Calmodulin Fraction
(μg Protein)

FIG. 16 Effect of Diabetes on Calmodulin activity in rat lung alveolar tissues. Activity was measured in the 15,000 x g supernatant and determined by its ability to activate a Calmodulin-deficient PDE. See Experimental Procedures for details. Control values of Calmodulin activity/mg protein were set at 100% ± SEM.

Fig. 16

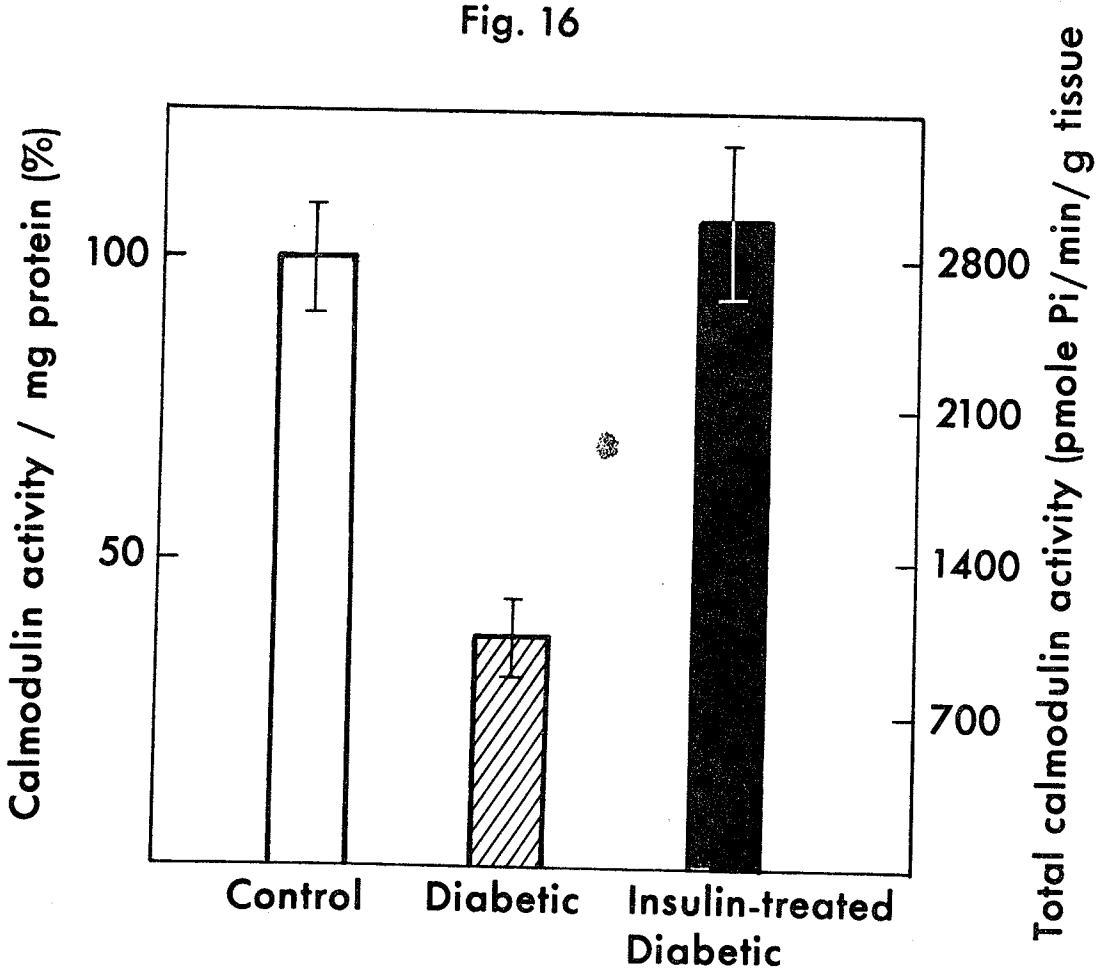

Calmodulin activity / mg protein (%)
100
50
2800
2100
1400
700
Total calmodulin activity (pmole Pi/min/g tissue
Control
Diabetic
Insulin-treated Diabetic

FIG. 17 Determination of a heat-stable PDE Inhibitory Protein in the 15,000 x g supernatant fractions from Control(●) and Diabetic(▲) rat lung alveolar tissues. The assays were performed at different concentrations of the PDE inhibitory protein preparations in the presence of Ca^{2+}-activatable PDE and 15ug of Calmodulin. See Experimental Procedures for details. Enzyme activity represents percentage of the activity of the PDE enzyme obtained in the absence of added PDE inhibitory protein samples. Each value is the mean of three separate experiments performed in triplicate samples.

Fig. 17

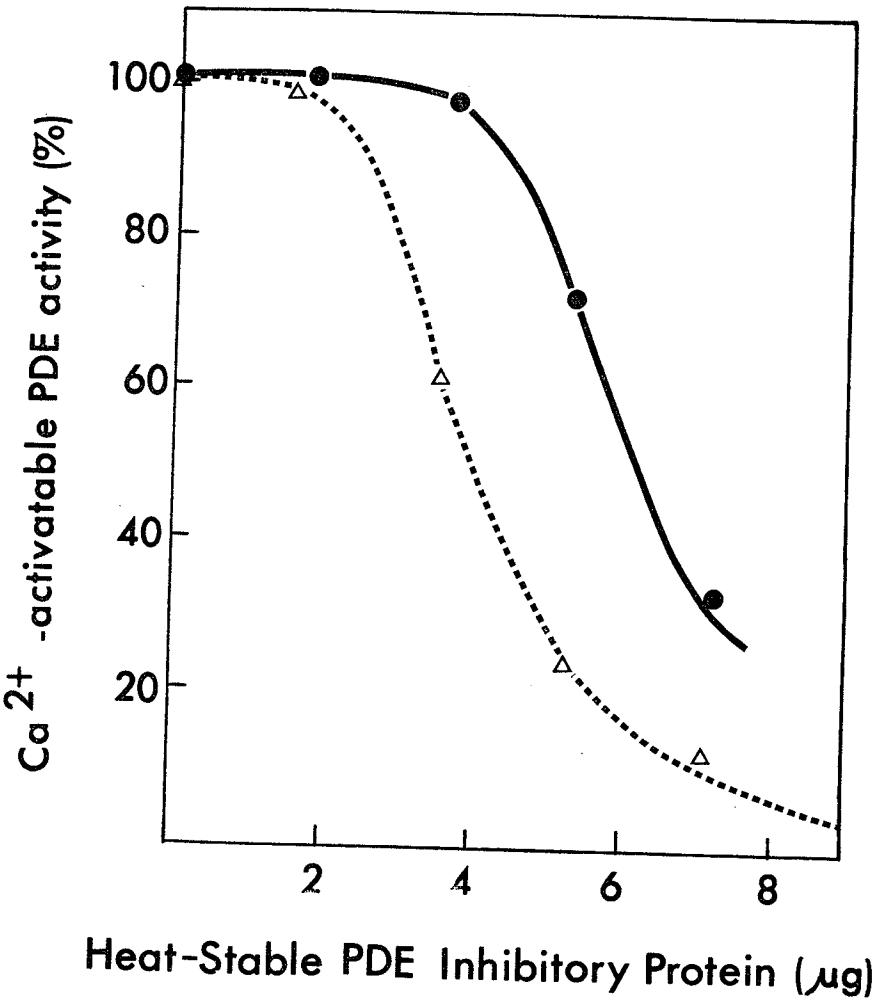

100
80
60
40
20
2
4
6
8
Ca 2+ -activatable PDE activity (%)
Heat-Stable PDE Inhibitory Protein (μg)

TABLE IX

INFLUENCE OF DIABETES ON THE ADENYLATE CYCLASE/CYCLIC AMP-PHOSPHODIESTERASE RATIO OF RAT LUNG ALVEOLAR TISSUES

The ratio of the activities of adenylate cyclase to cyclic AMP-Phosphodiesterase (pmole cAMP.min^{-1}/pmole Pi.min^{-1} g tissue^{-1}) was estimated as an index of the cyclic AMP content of the lung alveolar tissues. Values represent mean ± SEM of triplicate determinations from 3 different experiments performed with 10 rats in each group.

EXPERIMENTAL CONDITION	ADENYLATE CYCLASE / CYLIC AMP-PHOSPHODIESTERASE
CONTROL	8.37 ± 1.25
DIABETIC	20.75 ± 2.08
INSULIN-TREATED DIABETIC	11.42 ± 0.79

F. DISCUSSION AND CONCLUSION

The first striking features of this study are the significant reduction in absolute lung weight and the marked effect on lung protein and DNA content during streptozotocin-induced diabetes. Thurlbeck[170] had established that about 2 weeks after birth, the proliferation of rat lung cells is very active and new tissues are being rapidly laid down. There is a rapid increase in the amount of DNA, even though the amount of protein per nucleus remains approximately the same. After 2 weeks, lung growth enters a phase of cellular maturation and differentiation. The rate of cellular multiplication and formation of alveoli is markedly diminished. The lung grows by cellular expansion and the amount of protein per nucleus increases rapidly although the protein content per unit of lung weight remains fairly constant. This phase continues to approximately 5 to 7 weeks of age. In the final phase, cell proliferation stops, or is very slow, and cell enlargement is slight or may even stop; thus, the amount of protein per nucleus stays approximately constant, as does the amount of DNA. Hence in the present study, the demonstration that, although the lung protein concentration was unchanged, there was a marked increase in protein/DNA ratio coupled with the decrease in DNA content during diabetes is particularly important. Morishige et al[8] have reported similar findings;

however, these were attended to by a depressed ability of lung slices of the diabetic rats to incorporate (^{3}H)-leucine into acid-insoluble protein in vitro. The data presented therefore suggest the presence of more differentiated lung cells in diabetic rats. The reported increase in the protein/DNA ratio could be due to an increase in cell cytoplasm in tissues such as fibroblast and muscle and increase in intercellular connective tissue, since a general feature of the phase of cellular maturation and differentation during postnatal development of the lung is that of maturation of the interstitium[170].

Biochemical and morphologic studies of connective tissue components of various organs and tissues have indicated that, in diabetes, collagen and elastin undergo changes analogous to those which occur during aging[87-89]. Abnormalities in protein and collagen metabolism of fibroblasts from juvenile-onset and adult-onset diabetes have been described[92]. In this study, the diabetic alveolar lung tissue showed a higher hydroxyproline content than control. Madia et al[93] reported a similar change in diabetic rat lungs and demonstrated a 2-3 fold increase in lysyl oxidase activity, the enzyme which generates the collagen cross-linkage precursors. Hence the increase in collagen supports the earlier theory that the increased protein/DNA ratio in diabetic lung tissue may be due partly to an increase in intercellular connective tissue

that may result from a reduced turn-over of the connective tissue protein. Of particular interest, however, is the reported observation[4] that lung elastic recoil is significantly decreased in young men with juvenile-onset diabetes. Crystal[20] has emphasized that the stability of the lung alveoli depends partly on the elastin and collagen comprising the extracellular matrix of the alveolar septum; the elastic fibres are stretched at increasing lung volumes and in attempting to recoil require the supporting framework of collagen which produces the necessary stiffness that allows increasing recoil pressures at increasing lung volumes. It is therefore apparent that any condition which affects these processes will contribute to a decreased lung compliance. Although this study has not performed any biochemical measurement of the elastin content of lungs of diabetic animals, the indirect evidence presented here suggests that the decreased lung elastic recoil in diabetes is most probably not due to the increased collagen content; more collagen would allow a much higher increased recoil pressure at increasing lung volumes. Thus the possibility remains that loss of lung elastin in diabetes may be a major contributing factor in the decreased lung elastic recoil. However, the present study implicates the increased collagen content of the lung alveolar tissue as evidence of either a slow turn-over of the connective tissue protein or a reduced replicative capacity of the alveolar tissue fibroblasts.

The demonstration that the lung glycogen content was reduced in the diabetic animals is particularly important and assumes functional significance since it has been reported by Curle and Adamson[41] that lung glycogen during postnatal development is prominent in the mesenchymal cells, providing energy for rapid cellular multiplication. The data presented in this study correlates well with their finding. The depleted glycogen content of the diabetic lung tissue is coincident with differentiation of the fibroblasts, hence their reduced replicative capacity and therefore the increased synthesis of collagen. However, lung glycogen has another functional significance. It has been suggested that it plays a role in phospholipid synthesis by supplying both the glycerol and fatty acid portions of the phospholipid molecule[37]. Moxley and Longmore[36] have shown that glucose incorporation into lipids was increased by insulin treatment and decreased in experimental diabetes. The present study demonstrates a reduction in the phospholipid content of the lung tissues and of the surfactant fractions of tissues of the diabetic animals; the percent of phosphatidylcholine in the surfactant fractions was only slightly changed. Pathak et al[76] have reported a similar finding in Alloxan-induced diabetic rat lungs. Of particular interest in the present study was the finding that during diabetes there was a reduction in the amount of disaturated phosphatidylcholine and of lyso-phosphatidylcholine in the total surfactant phosphatidylcholine. This finding

correlates well with the studies of Rhoades et al[71]. They observed that the enzyme activities in the CDPcholine pathway were elevated while the percent disaturated phosphatidylcholine was decreased in rat lungs during neonatal diabetes, and suggested that pathways other than those responsible for phospholipid synthesis, such as the deacylation and reacylation enzymes (responsible for the remodeling of the phosphatidylcholine molecules), may be affected during diabetes. A Ca^{2+}-dependent phospholipase A_2 activity has been reported in the rat lung particulate fractions[68,69]. Phospholipase A_2 is the enzyme responsible for deacylation of phosphatidylcholine molecules into lyso-phosphatidylcholine molecules; the lyso-phosphatidylcholine molecules can then undergo reacylation with a saturated fatty acid (palmitic acid) to form disaturated phosphatidylcholine molecules, the principal agent responsible for surface tension reducing properties in the surfactant complex[54]. In the present study, the observed reduction in the activity of Calmodulin, a regulatory protein functioning as a mediator of Ca^{2+} effects[145], in the diabetic rat lung particulate fractions provides the preliminary evidence that the depressed level of disaturated phosphatidylcholine in the surfactant complex may be due to a reduction in calmodulin activity and therefore a reduction in the Ca^{2+}-dependent phospholipase A_2 activity in the particulate fractions of the lung tissues. The decrease in phosphatidylglycerol could be due to reduction in the utilization of glucose for phospho-

lipid synthesis as indicated by the glucose perfusion experiments of Moxley and Longmore[7]. It is important to mention that the present study supports the considerable body of evidence[19,54,72,73] which indicate phosphatidylglycerol and disaturated phosphatidylcholine as the major defective components of the surfactant complex apparent in the diabetic lung that may lead to the respiratory distress syndrome.

Biochemically, this study presents evidence that the lung alveolar Type II cells are affected in diabetes. A considerable body of evidence indicates the alveolar Type II cells as the site of synthesis and storage of the surfactant complex[42,58,59]. The protein and phospholipid components are synthesized by the endoplasmic reticulum, packaged in the golgi apparatus and then stored in the lamellar bodies until secretion of the surfactant onto the alveolar surface is stimulated[62-64]. Ultra-structurally, this study has provided evidence to believe that the depressed metabolic activity of the alveolar Type II cells is partly due to a defect in the normal function of the granular endoplasmic reticulum of the Type II cell. Plopper and Morishige[96] have indicated that special attention should be given to the interaction of endoplasmic reticulum function and insulin, since in their study this organelle was the most dramatically altered by streptozotocin-induced diabetes and was the most responsive to

insulin therapy. However, this study has demonstrated another major alteration in the Type II cells during diabetes, which is the disintegration of the mitochondrial cristae. This finding correlates well with the reports of Rhoades et al[71] that during diabetes there is a reduction in glucose oxidation to CO_2, a function associated with the mitochondria. Thus the depressed metabolic activity of the alveolar Type II cells during diabetes may also be due to a defect in the normal function of the mitochondria, an organelle responsible for the generation of metabolic energy[29-31].

In the present study, a reduction in the basal adenylate cyclase activity was demonstrated in the particulate fractions of diabetic rat lung tissue. A similar finding has been reported in adipose tissue of diabetic rats[158]. The decrease of basal adenylate cyclase activity may be the result either of a diminished enzyme content and/or of an altered functional state of the enzyme. The results presented in this study demonstrated the presence of calmodulin in lung tissue and the existence of a Ca^{2+}-dependent adenylate cyclase in the particulate fraction which is dependent on calmodulin for the expression of its basal activity (Tables V and VI, Figure 11). Since a decrease in calmodulin activity in the particulate fraction of diabetic lung tissues was demonstrated, this study assumes that the decrease in basal adenylate cyclase activity is due to an altered functional state that

may have resulted from the loss of calmodulin. However, Nijjar[121] has reported that cytoplasmic factor(s) in lungs play a regulatory role in the maximal activation of the particulate adenylate cyclase. The data presented in this study support his observation. The evidence is in the findings that the tissue homogenates of each experimental group present higher specific adenylate cyclase activities when compared with the basal adenylate cyclase activities in the particulate fractions. The present study, however, observed that in diatetes the supernatant fraction activated the particulate adenylate cyclase to a much higher extent that in normal circumstances. Since the activation was independent of the particulate fraction used, it is assumed that the increased activation of particulate adenylate cyclase in the diabetic lung is due to an increase in the activity of the cytoplasmic factor(s) and not necessarily related to a possible increase in the response of the particulate adenylate cyclase to the cytoplasmic factor(s).

Nijjar[121] has suggested that the cytoplasmic factor(s) may have a role in cellular differentiation. Earlier, the present study had implied that during diabetes there appears to be more differentiated lung cells, possibly differentiated fibroblasts which may account for the increased collagen content of the diabetic lung. The finding in this study of a higher increase in the activity of the cytoplasmic

factor(s) in rats rendered diabetic prior to the time of cessation of rapid alveolar multiplication than in rats rendered diabetic thereafter is particularly interesting. It suggests that diabetes may accelerate cellular differentiation of the lung tissue by increasing the activity of the cytoplasmic factor(s). Diabetes has been described as a premature aging process, in the sense that there is a reduction in the in vitro replicative capacity of cells obtained from diabetic patients[171]. Thus this study provides the first supportive evidence that the lung cytoplasmic factor(s) do have a role in cellular differentiation.

Though reports in other tissues[109-113] have indicated the presence of cytoplasmic activators of adenylate cyclase, the possibility has been raised that the lung cytoplasmic factor(s) may be calmodulin or calmodulin bound to another protein. Cheung[145] has defined a set of criteria to ascertain whether a given reaction is calmodulin regulated. First, the tissue or cell should possess sufficient calmodulin in the appropriate locale. Second, since calmodulin requires Ca^{2+} for activity, sequestering $Ca^{2}+$ from the reaction system by an appropriate chelator, such as EGTA, should return the calmodulin-induced activity to the basal level. Third, depletion of endogeneous calmodulin by appropriate means should alter the activity of the reaction in question. Fourth, calmodulin in the presence of Ca^{2+} avidly binds trifluoperazine;

upon binding, calmodulin becomes biologically inactive. Thus the addition of this compound should return the calmodulin-dependent activity to the steady-state level. However, the hydrophobic nature of trifluoperazine makes it important to ascertain whether its effect on the test system is due to its interaction with hydrophobic environments or to its specific action on calmodulin. On the basis of the first three criteria, the present study has evidence to assume that the activation of basal adenylate cyclase by the supernatant fraction of rat lung is not due to the presence of calmodulin in the supernatant fraction. Despite the demonstration of a sufficient activity of calmodulin in the supernatant fraction of the lung tissues, the independent additions of EGTA and calmodulin-binding protein to the assay system failed to reduce the supernatant activation of particulate adenylate cyclase to the basal level of activity. Calmodulin is a single polypeptide with a molecular weight of 16,700 daltons[145]. According to the present findings in Nijjar's laboratory (Nijjar, privileged communication), the cytoplasmic factor(s) responsible for the supernatant activation of lung particulate adenylate cyclase comprise of two proteins, one of low molecular weight (14,000-15,000 daltons) and the other of high molecular weight (65,000 daltons). It appears that both components function in a cooperative manner. However, it is important to stress that the molecular weight reported for calmodulin is that of bovine brain calmodulin,

where calmodulin exists as an independent protein molecule[145]. Lung supernatant calmodulin has been reported to be tightly bound to phosphodiesterase[136]. Moreover, the supernatant fraction used in the present study was not dialyzed against EGTA prior to use as was performed in the investigation with the particulate fraction; it appears that this procedure is necessary in order to ascertain the calmodulin dependence of a reaction. Also, maybe the added calmodulin-binding protein in the assay system was not sufficient enough to exert its effect. Thus the assumption here that the activation of basal adenylate cyclase by the supernatant fraction of rat lung is independent of calmodulin should be cautionarily accepted; further investigations are necessary to substantiate the reported observations. Nonetheless, the data presented in this study shows that the increased activation of the particulate adenylate cyclase by the supernatant fractions in the diabetic lung is due to an increase in the amount of the 65,000 dalton protein, since this was the only protein component altered during the streptozotocin-induced diabetes. Thus the high molecular weight protein may be implicated for the accentuated cellular differentiation observed in the diabetic rat lung tissue.

It is becoming increasingly apparent that the lung tissue is dependent on insulin for normal metabolic function[4-10]. Insulin lowers cyclic AMP concentrations in

stimulated liver and fat cells by apparently activating a low K_m membrane-bound PDE[127,148]. The data presented in this study show that during diabetes, lung cyclic AMP-PDE activity becomes depressed. Interesting in the present investigation was the observation that there appears to be no difference in the nature of cyclic AMP-PDE present in the particulate and supernatant fractions; both have Ca^{2+}-independent and Ca^{2+}-dependent activities. In the particulate fraction both activities were depressed, whereas in the supernatant fraction only the Ca^{2+}-dependent activity was afffected. Insulin deficiency during diabetes explains the depressed activity of the particulate Ca^{2+}-independent cyclic AMP-PDE, since this is the activity that corresponds to the insulin sensitive PDE in liver and fat cells[127,148]. However, there is possibility that alteration of calmodulin activity may contribute to the depression of lung cyclic AMP-PDE during diabetes. Evidence presented in the present study appears to suggest that in diabetes there is a translocation of calmodulin from the particulate to the supernatant fraction. Similar translocations have been observed in other tissues[150,172]. This finding may account for the decreased activity of the particulate Ca^{2+}-dependent cyclic AMP-PDE but fails to explain the situation in the supernatant fraction. Overall, the present study has shown that during diabetes there was a reduction in the activity of lung tissue calmodulin. Expression of the data as calmodulin activity per mg protein and as total calmodulin

activity is assuring that the decrease in calmodulin activity was real and not related merely to a nonspecific decrease in the tissue protein content that may occur in experimental diabetes[84]. A heat-stable inhibitor of PDE has been identified, in brain tissue, which lowers the V_{max} of PDE by binding to calmodulin without altering the K_m[141]. The present study has shown that an inhibitor of Ca^{2+}-activatable cyclic AMP-PDE activity is present in rat lung tissue and that the activity of this inhibitor is increased in diabetes. However, further analysis would be necessary to elucidate the nature of this heat-stable inhibitor. Meanwhile, the finding provides an explanation for the reduced Ca^{2+}-dependent cyclic AMP-PDE activity in the diabetic lung alveolar tissue despite the translocation of calmodulin from the particulate to the supernatant fraction.

Cyclic AMP has been ascribed a fundamental regulatory function in mammalian cells[143]. In many instances, the effects of cyclic AMP and Ca^{2+}-calmodulin complex are often intertwined, that is, one may accentuate or attenuate the effect of the other[145]. The present study has utilized the ratio of the activities of adenylate cyclase and cyclic AMP-PDE as an index of the cyclic AMP level. On this basis, it appears that during diabetes there is an increase in lung tissue cyclic AMP concentration. Such an increase has been shown in diabetic fat and liver cells[173]. Morishige et al[8] demon-

strated insulin receptors in membrane preparations of normal rat lung. The findings in the present study show that these membrane preparations containing adenylate cyclase also contain insulin sensitive PDE, Ca^{2+}-dependent PDE and calmodulin. Thus the localization of these enzymes and modulator may be the most significant factor determining the depressed pulmonary activity in diabetes mellitus.

The findings presented in this study provide the following tentative but unifying hypothesis. The lack of insulin in diabetes causes reduced stimulation of the insulin-sensitive PDE and therefore less hydrolysis of tissue cyclic AMP. A transient increase in the concentration of cyclic AMP may cause increased tissue protein degradation providing cells with amino acids to synthesize new proteins appropriate to the new metabolic condition. The proteolysis may also cause the conversion of inactive proteins into the active forms. These account for both the increase in the amount and activity of the cytoplasmic factors which in diabetes increases the activation of the particulate adenylate cyclase. The cyclic AMP level is increased further causing membrane-associated calmodulin-binding sites to be altered by phosphorylation and thus releasing calmodulin to the cytoplasm. The release of calmodulin from the membrane causes a reduction in the activity of the particulate Ca^{2+}-dependent phospholipase A_2 and therefore reduced remodeling of PC molecules

into disaturated PC, the principal agent responsible for surface tension reducing properties of the surfactant complex. Calmodulin released to the cytoplasm should have served to activate the soluble Ca^{2+}-dependent PDE but the increased activity of the inhibitory protein of Ca^{2+}-activatable PDE in diabetes prevents this. Hence the increased cyclic AMP level remains, and may provide for the increase in the differentiated state of the lung tissue. However, the cyclic AMP fails to account for the reduced total phospholipid content of diabetic lung tissue. Cyclic AMP has been implicated to stimulate glycogenolysis[11] and therefore the provision of substrate for phospholipid synthesis[37,174]. In the absence of experimental evidence, it is assumed that the cyclic AMP is functioning in glycogenolysis during diabetes as shown by the depletion in lung glycogen content but there is a reduced flow along the pathways leading from pyruvate as reported by Morishige et al[8]. Thus, though the activity of the pentose pathway, the pathway that generates the reducing equivalents that function as cofactor in phospholipid and fatty acid synthesis, is normal in diabetes[25], the intracellular supply of free fatty acid may be limiting. This hypothesis correlates well with the findings of Das and Kumar[5], and provides an explanation for the decrease in phosphatidylglycerol in the surfactant fraction of the diabetic lung. However, the possibility remains that cyclic AMP may be involved in the degradation of pulmonary phospholipds[175].

In summary, this study presents evidences that the altered and/or uncoordinated activities of the enzymes and endogenous modulators related to the metabolism of cyclic AMP and of calmodulin may be the most significant factors responsible for the depressed pulmonary activity in diabetes mellitus. However, further investigation would be necessary in order to clearly define the biochemical basis of lung dysfunction in diabetes.

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