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Comparison of Protein Kinase Activity in Tissue Extracts of the Diabetic and Nondiabetic Chinese Hamster

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COMPARISON OF PROTEIN KINASE ACTIVITY IN TISSUE
EXTRACTS OF THE DIABETIC AND NONDIABETIC
CHINESE HAMSTER

by

Michael Andrew Dombos

A Thesis
Submitted to the
Faculty of the Graduate College
in partial fulfillment
of the
Degree of Master of Arts

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Michael Andrew Dombos

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INTRODUCTION

Protein kinases may act as third messengers in the regulation of glycogenolysis, glycogen synthesis, lipolysis, and protein synthesis (68). The phosphorylation of phosphorylase kinase (76), glycogen synthase (77), and hormone-sensitive triglyceride lipase (37,38) is mediated by a cyclic AMP-stimulated protein kinase. These phosphorylated enzymes regulate glycogen breakdown and synthesis and lipolysis. Phosphorylase kinase and lipase are activated by phosphorylation while glycogen synthase is inactivated by phosphorylation. Since lipid, protein and carbohydrate metabolism are abnormal in the diabetic, hyperglycemia and other metabolic abnormalities may be associated with an aberrant protein kinase system. Moreover, hormone and cyclic AMP concentrations affect glycogenolysis and gluconeogenesis through protein kinases. For example, glucagon and catecholamines, by increasing cyclic AMP production, and exogenous cyclic AMP activated glycogenolysis and gluconeogenesis in perfused rat livers. In contrast, insulin decreased intracellular cyclic AMP levels and antagonized the effects of glucagon, catecholamines, and exogenous cyclic AMP causing a decrease in glucose output (22). This suggests that insulin decreased glucose production by lowering cyclic AMP levels in liver. The hyperglycemic, hypoinsulinemic diabetic animal may exhibit high cyclic AMP levels and consequently a more active protein kinase which in turn increases basal gluconeogenesis. This metabolic activity would contribute to the abnormally high blood

sugar levels in diabetic animals. In summary, protein kinase activity is affected by changes in cyclic AMP concentrations. Glucagon and catecholamines increase cyclic AMP concentrations and protein kinase activity and insulin decreases cyclic AMP concentrations and consequently protein kinase activity.

An abnormal protein kinase system might be casually related to the abnormal metabolism found in the diabetic since increased basal and/or cyclic AMP-stimulated protein kinase activity in the liver might be expected to increase glycogenolysis and gluconeogenesis. This breakdown of glycogen and increase in the production of glucose could contribute to abnormal blood sugar levels in the diabetic animal.

A hyperinsulinemic or hypoinsulinemic state could result in abnormal protein kinase activity. In the former, high levels of plasma insulin reduces intracellular cyclic AMP concentrations presumably by stimulating phosphodiesterase (18,78). Thus basal protein kinase activity in the hyperinsulinemic animal would be decreased due to the depressed cyclic AMP concentration. This appears to be the case in adipose tissue of the db/db mutant of the C57BL/KsJ mouse. These diabetic mice are a hyperinsulinemic, obese line during the early part of their life span but become hypoinsulinemic later in life. Kupiecki and Adams (46) reported that the young hyperinsulinemic db/db mouse exhibited lower in vitro basal protein kinase activity in epididymal fat pads compared with controls. Thus high plasma insulin levels may have reduced cyclic AMP levels in the db/db mice with a concomitant decrease in basal protein kinase activity.

Also they reported an elevation in in vitro cyclic AMP-stimulated protein kinase activity in epididymal fat pads in these mice. The increase in cyclic AMP-stimulated activity may suggest an in vivo compensatory increase in protein kinase synthesis in these animals allowing them to function better in spite of decreased cyclic AMP levels. Thus when protein kinase activity was measured in vitro the db/db mouse exhibited greater activity when compared with the control. If this increased cyclic AMP-stimulated activity also occurred in vivo in liver, gluconeogenesis and glycogenolysis would be activated with a concomitant elevation in blood sugar.

When plasma insulin levels are depressed it is conceivable that cyclic AMP levels would increase because insulin would not inhibit cyclic AMP formation. Jefferson et al. (40) reported that in livers from alloxan-diabetic rats cyclic AMP levels were increased two-fold over nondiabetic controls. An increase in basal protein kinase activity in this type of diabetic animal might be expected when compared with nondiabetic controls. Thus, increased protein kinase activity in the diabetic would activate gluconeogenesis and glycogenolysis and would contribute to an elevation in blood sugar.

Insulin not only regulates intracellular cyclic AMP levels but it also appears to influence protein kinase synthesis. Zapf and his associates measured protein kinase activity in vitro in nondiabetic and streptozotocin-induced diabetic rats. Their data indicated that in the liver from the diabetic the basal and cyclic AMP-stimulated activity was decreased by 35%. Basal and cyclic AMP-stimulated activity in adipose tissue from diabetics was reduced by 30%. They

suggested the decrease was due to depressed protein kinase synthesis which resulted from insulin deficiency (94). However, other work by Zapf et al. (93) showed no consistent tissue-to-tissue change in basal or cyclic AMP-stimulated protein kinase activity caused by diabetes. They examined adipose tissue, liver tissue, heart, and skeletal muscle from streptozotocin-induced diabetic rats in this study. This suggests that depressed protein kinase synthesis resulting from insulin deficiency may not account for the differences in protein kinase activity in all tissues.

Weber et al. (89) suggested that the insulin/glucagon ratio was important in regulating protein kinase activity. Their studies showed that in liver tissue from alloxan-induced diabetic rats cyclic AMP-stimulated protein kinase activity was decreased. In the diabetic animals the insulin/glucagon ratio was decreased. This might suggest that in vivo basal protein kinase activity would not be well regulated as a result of low insulin levels. Consequently, basal protein kinase activity might keep gluconeogenesis and glycogenolysis active. This system would account for the decreased cyclic AMP-stimulated protein kinase activity measured in the diabetic since a portion of the protein kinase in the diabetic would already be activated in the basal state.

The investigations described in this thesis, using the diabetic Chinese hamster as the experimental animal, were an attempt to determine if any differences exist in the protein kinase activities in various tissue extracts. This information could provide clues on factors causing abnormalities in carbohydrate, lipid and protein

metabolism in the diabetic animal. This experimentation was divided into two parts. The first part involved the determination of some assay conditions for protein kinase in tissues of the Chinese hamster. This involved evaluating the effect of ATP concentration and substrate preference with tissue homogenates. The second part of this thesis involved a comparison of protein kinase activity in the diabetic and nondiabetic Chinese hamster.

The assumption was made that protein kinase activity measured in vitro with non-enzyme substrates is a valid measure of in vivo protein kinase action on endogenous enzyme substrates. This assumption which has been made by others appears to be reasonable. For example, Walsh et al. (87) showed that a partially purified protein kinase from rabbit skeletal muscle phosphorylated phosphorylase kinase as well as protamine and casein. More recently, Singh and Wang (73) showed that a cyclic AMP-dependent protein kinase from rabbit skeletal muscle phosphorylated phosphorylase kinase, casein and histone to a similar extent.

LITERATURE REVIEW

The clinical symptoms of diabetes mellitus were recorded over 3500 years ago in Egyptian culture (24). However it was not until this century that any real progress was made in describing the metabolic and morphological abnormalities and consequences of the diabetic states. Diabetes mellitus is commonly characterized as a metabolic disorder associated with relative or absolute insulin deficiency. This insulin deficiency manifests itself by impairing lipid, protein and carbohydrate metabolism.

Although many morphological and metabolic changes in diabetics have been described, we are still unable to prevent its occurrence. Renold and Dulin (60) have suggested that any further understanding of the diabetic syndrome is dependent on progress in four critical areas. These are 1) knowledge of genetic transmission, 2) number of primary defects, 3) relation of environment and diabetes, and 4) relationship of diabetes to metabolic and morphological abnormalities.

The importance of finding answers to these questions is obvious since diabetes with its complications ranks behind heart disease and cancer as the third major cause of death in the United States (96). The percentage of diabetics in the population of the United States increased 50% between 1965 and 1973 so that now diabetes affects five percent of the total population. Today there is better than a one in five chance that a baby born in the United States will eventually develop diabetes (96).

The study of diabetic metabolic disorders and their progression in man have been difficult. No screening method for diabetes has been developed to distinguish persons predisposed to diabetes. Consequently researchers have studied diabetic disorders after the metabolic abnormalities have become manifest. Research in retardation and reversal of diabetic symptoms at this stage is difficult if not impossible. Although the nondiabetic identical twin with a diabetic twin sibling is genetically predisposed to develop diabetes, the development of diabetic symptoms in the nondiabetic twin may evolve over a long period of time if they evolve at all. Moreover, genetic, cultural and environmental parameters are impossible to regulate for human subjects (20,21). These considerations make the study of prevention, progression and retardation of biochemical and morphological complications associated with diabetes extremely difficult. Therefore an animal model exhibiting a comparable mode of inheritance and diabetic symptoms as well as a short life span would be extremely valuable. An animal model would allow for controlled environmental, dietary and breeding studies. Three symposia (61, 62,63) on spontaneous diabetes in laboratory animals and several reviews (1,74,81) on diabetes in animal models have been presented in the literature.

Chinese Hamster

The Chinese hamster (Cricetulus griseus) may be the most suitable animal model with which to study diabetes. Genetic diabetic and nondiabetic lines have been developed. Moreover a prediabetic

animal can be produced by mating two ketotic animals. These pre-diabetics, diabetically asymptomatic, will eventually develop diabetes within a specific time interval. Secondly, the Chinese hamster exhibits a diabetes which is similar to the diabetes observed in man (21,24).

A polygenic inheritance scheme has been proposed for the Chinese hamster (7,8,21) similar to that proposed for man. The time of onset is variable (67) and both sexes are affected (32). Beta cell granulation is decreased in the diabetic (9,50,51,56,72,80). Insulin synthesis in vivo is also decreased in the diabetic hamster (10). Insulin response to a glucose stimulus in the diabetic hamster is decreased in vivo (25,30) and in vitro (23,35). Plasma and pancreatic insulin is generally decreased in the diabetic (19,20,35,52,91). The hepatic gluconeogenic enzymes are increased in the diabetic while the glycolytic enzymes are decreased (11). Glucose production from pyruvate is increased in the diabetic animal (11). The glucose utilization in muscle and lipid are the same for both diabetic and nondiabetic animals (20,30) and the hamster is not obese (25). Similar morphological changes to those observed in human diabetics have been observed in diabetic Chinese hamsters. This includes changes in the retina (24, 79,80), kidney (69,70,80), nervous system (53,66), vascular system (52), and skeletal system (70,71). Likewise, metabolic changes have been observed in the vascular system (12,13). Like man, mild diabetics respond well to sulfonylureas while severe diabetics do not (29). Restricted diet and diet content can ameliorate diabetic symptoms

and severity (21,24,25,26,27,31). Death in the diabetic hamsters has been associated with complications arising from ketosis (32).

Protein Kinase

Hormones secreted by specific tissues are released into the vascular system exerting their metabolic effects on a specific target tissue. Since polypeptide hormones are incapable of penetrating the receptor cell membrane in the target tissue the message must be transmitted to the interior by some other method (34,49,84). One such method by which these intracellular metabolic effects are initiated in the target tissues is by stimulating the production of cyclic AMP (34,49,68).

Sutherland and his associates described cyclic AMP as a second messenger, since the hormone (the first messenger) after interacting with the receptor sites in the plasma membrane would produce a change in the intracellular levels of cyclic AMP. Because this change in intracellular levels of cyclic AMP was related to subsequent metabolic changes, cyclic AMP was characterized as an intracellular messenger or a second messenger (34,49,59,84).

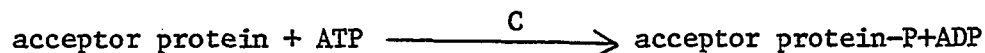
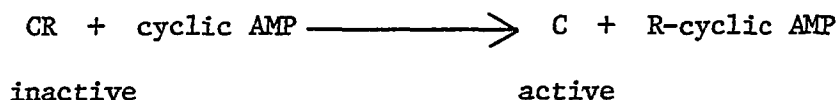
Cyclic AMP is synthesized from ATP by the adenylate cyclase system in the cell membrane. Cyclic AMP consists of: a base, adenine; a five-carbon sugar, ribose; and a phosphate group attached to the 3' and 5' position on the sugar moiety. An enzyme, phosphodiesterase, inactivates cyclic AMP by breaking the phosphate-ribose ring to form 5'AMP. This degradation process can be inhibited by phosphodiesterase inhibitors as caffeine and theo-

phylline (34,49).

Cyclic AMP-dependent protein kinases are involved in many systems which are activated by cyclic AMP such as the phosphorylase kinase glycogen synthetase, and hormone-sensitive triglyceride lipase systems (37,48,45,54,68,77). Reviews have described protein kinases in various vertebrate and invertebrate species and tissues (44,45). Protein kinase activity has also been reported in bacteria (43) and in Rauscher Murine leukemia virus (83).

Cyclic AMP-dependent protein kinases act as a link between the cyclic AMP produced by the adenylate cyclase system and the cellular metabolic changes. When activated, protein kinase activates other enzymes by transferring the gamma phosphate from ATP to serine or threonine residues (34,42,49,68,85). It is these phosphorylated enzymes which then modify specific cellular activity.

Recently, many investigators have attempted to elucidate the mechanism through which cyclic AMP exerts its effect on intracellular metabolism via protein kinases. These investigations indicate cyclic AMP-dependent protein kinases consist of two subunits: a catalytic (C) subunit and cyclic nucleotide binding (R) subunit. The enzyme is activated when the cyclic nucleotide binds to the R subunit releasing the C subunit. Thus, the binding causes the dissociation of the holoenzyme into two subunits and thereby the release of the catalytic subunit from inhibition. The proposed scheme by which this dissociation occurs is diagrammed below (2,3, 14,15,17,33,34,41,42,49,59,82,85).



Proteins which serve as substrates for mammalian protein kinase include histones (3,14,16,34,42,47,57,86), protamine (34,45,57,85,86, 87), casein (3,14,16,34,86,87), phosphorylase kinase (14,16,33,68, 77,85,87), glycogen synthetase (14,65,76), and hormone sensitive lipase (38,68). When phosphorylase kinase and hormone sensitive lipase are phosphorylated they are converted from an inactive form to an active form. While phosphorylation of glycogen synthetase converts the enzyme from an active to an inactive form.

The following section is a brief review of literature on the metabolic significance of protein kinases in a number of mammalian tissues and species. Evidence describing the mechanism through which cyclic AMP activates these protein kinases will be cited. Cyclic AMP-dependent protein kinase activity was examined in five tissues (heart and skeletal muscle, liver, brain and epididymal fat) in my research. Therefore this literature review will emphasize significant investigations which have contributed to elucidating protein kinase activity in these tissues.

Brain

An adenosine 3',5'-monophosphate dependent kinase was extracted from rabbit brain by Miyamoto et al. (57). Miyamoto and his colleagues partially purified a crude protein kinase extract from brain and obtained a 150-fold increase in specific activity. A comparison of substrates

for the partially purified protein kinase showed protamine sulfate to be more effective as a phosphate acceptor than either histone or phosvitin.

Liver

Sutherland and Rall (84) cited evidence for the activation of phosphorylase kinase by cyclic AMP in the liver. Furthermore they suggested that this activation was mediated by a protein kinase which transferred a phosphate group from ATP to inactive phosphorylase kinase and thereby activated it.

Langan (47) purified a protein kinase from liver homogenates 150-fold. With this protein kinase, he obtained a four-six fold increase over crude extracts when histone was used as the substrate.

Kumon et al. (41,42) showed that the cyclic AMP-dependent and independent protein kinases obtained from rat liver were different forms of one kinase. They obtained three fractions using a dialyzed calcium phosphate gel eluate passed through a DEAE cellulose column. One fraction was the holoenzyme and was stimulated by cyclic AMP. The other two fractions were the regulatory (R) subunit and the catalytic (C) subunit. This work suggested that the holoenzyme was actually a complex of the regulatory subunit and catalytic subunit. In addition, these results suggested that cyclic AMP was involved in its regulation and activation.

Cyclic AMP-dependent protein kinase activity in liver homogenates from streptozotocin-induced diabetic rats was reported by Zapf and his associates. Their work was mainly concerned with characterizing some of the protein kinase parameters. For example, protein kinase phos-

phorylation of casein in the presence of 10^{-5} M cyclic AMP was linear for 7-10 minutes. However, diabetic liver homogenates compared with nondiabetic homogenates exhibited a 35% decrease in basal and cyclic AMP-stimulated activity and a 30% decrease in cyclic AMP binding activity. They suggest this decrease could be attributed to depressed protein kinase synthesis which resulted from insulin deficiency (94). More recently (93), they have suggested that the basal protein kinase activity was not significantly different in diabetic and nondiabetic animals but that cyclic AMP-stimulated activity was reduced.

Protein kinase activity in liver homogenates from alloxan-induced diabetic rats was examined by Weber et al. (89). They found cyclic AMP-stimulated activity was lower in the diabetic animal compared with nondiabetic controls. Their data further suggested that the insulin/glucagon ration was important in regulating protein kinase activity. Low insulin levels might allow gluconeogenesis and glycolysis to remain active. Since a portion of the protein kinase in the diabetic might be activated, cyclic AMP-stimulated activity would be lower.

The protein kinase system appears to be aberrant in these diabetic animals. This abnormality in the protein kinase may therefore contribute to an abnormal metabolism in the diabetic.

Skeletal and cardiac muscle

Glycogenolysis in skeletal muscle has been characterized extensively. Glycogen breakdown to glucose is activated by epinephrine

and glucagon via receptors in the cell membrane. Intracellularly, glycogenolysis is mediated through an enzyme called phosphorylase. Phosphorylase exists in an active (phosphorylase b) and an inactive (phosphorylase a) form. Activated phosphorylase in the presence of ATP phosphorylates the [1 \rightarrow 4] glycosidic linkage yielding glucose-1-phosphate leaving the glycogen chain with one less glucose moiety. Phosphorylase activation in turn is mediated by a phosphorylase kinase. Phosphorylase kinase like phosphorylase exists in two forms: an active form (phosphorylase a kinase) and an inactive form (phosphorylase b kinase). The activated phosphorylase kinase catalyzes the transfer of the gamma phosphate from ATP to phosphorylase and thereby activates phosphorylase. This phosphorylase kinase is activated by a cyclic AMP-dependent protein kinase(s). Cyclic AMP-dependent protein kinase(s) is stimulated by increased levels of cyclic AMP. In summary, when cyclic AMP levels rise via epinephrine or glucagon interaction with the cell membrane, the protein kinase activates phosphorylase by transferring the gamma phosphate from ATP to phosphorylase kinase (49,54,68,84).

More recently, experimental evidence has characterized these protein kinases in skeletal and cardiac muscle. The mechanism through which protein kinase(s) is activated in skeletal and cardiac muscle will be discussed.

Walsh, Perkins, and Krebs (87) characterized a partially purified adenosine 3',5'-monophosphate-dependent protein kinase from rabbit skeletal muscle. These researchers suggested that this cyclic AMP-dependent protein kinase might serve as a link between hormonally

activated adenylyl cyclase and phosphorylase kinase. This cyclic AMP-dependent protein kinase catalyzed the transfer of the labelled gamma phosphate from ATP to casein and protamine. Protamine phosphorylation occurred at a rate five to eight times greater than observed with casein. Other cyclic and noncyclic nucleotides were unsuccessful substitutes for cyclic AMP. Phosphorylation of phosphorylase kinase was enhanced when the partially purified cyclic AMP-dependent protein kinase was incubated with phosphorylase kinase.

Reimann et al. (59) and Brostrom et al. (3) provided evidence on the mechanism through which cyclic AMP activated protein kinase by causing a dissociation of the holoenzyme into a regulatory subunit (R) and a catalytic subunit (C) in rabbit skeletal muscle. Reimann and his group (59) showed that the holoenzyme separated into catalytic and regulatory subunits in the presence of cyclic AMP. Cyclic nucleotide dependence was restored with the addition of the regulatory subunit to the catalytic subunit. Brostrom and his coworkers (3) presented evidence suggesting that when the holoenzyme dissociated in the presence of cyclic AMP the regulatory subunit was in the form of an R-cyclic AMP complex. The addition of the catalytic subunit to the R-cyclic AMP complex released the bound cyclic AMP.

Protein kinase activity in skeletal muscle from nondiabetic and streptozotocin-induced diabetic rats has been reported (93). Although cyclic AMP-stimulated protein kinase activity in skeletal muscle was the same in diabetic and nondiabetic, basal protein kinase activity was lower for the diabetic animal. They suggested protein kinase was stimulated to a greater extent with cyclic AMP in the

diabetic animal. This abnormal protein kinase stimulation then may have an effect on the metabolism in the diabetic animal.

Cyclic AMP-stimulated protein kinase has been implicated in both chronotropic and inotropic effects of catecholamines and also in the regulation of glycogenolysis in the heart. It has been suggested that the regulation of glycogenolysis in cardiac muscle and skeletal muscle is similar (49,65,68,84).

Brostrom et al. (2) obtained a 250-fold purification of a cyclic AMP-stimulated protein kinase from bovine cardiac muscle using ammonium sulfate fractionation and column chromatography. This protein kinase phosphorylated casein and histone. The reaction was specific for and was enhanced by cyclic AMP compared with other cyclic nucleotides. More recently, two protein kinases from bovine heart muscle have been resolved (65). The partially purified protein kinase could be resolved into two protein kinases via polyacrylamide gel electrophoresis, ultracentrifugation or storage at 4°C. Both forms were cyclic AMP-dependent and were composed of the same two kinds of subunits. These investigations have provided information which suggest that in heart and skeletal muscle cyclic AMP activates protein kinase. They further suggest that cyclic AMP binding to the regulatory subunit releases the catalytic subunit from inhibition.

Zapf's work with protein kinase activity in heart muscle from nondiabetic and streptozotocin-induced diabetic rats indicated that cyclic AMP-stimulated activity was decreased compared to the non-diabetic. Whereas basal protein kinase activity was not significantly different in either group (93). This difference in the protein

kinase system in the diabetic may contribute to its abnormal metabolism.

Epididymal fat pads

Cyclic AMP-dependent protein(s) are involved in the regulation of lipolysis in adipose tissue. Initial studies showed that insulin had an antilipolytic effect on adipose tissue by lowering intracellular cyclic AMP levels whereas epinephrine had a lipolytic effect by increasing intracellular cyclic AMP levels (5,6,95). These changes in cyclic AMP levels influenced the activity of hormone-sensitive lipase. This lipase exists in an active and an inactive form. When cyclic AMP levels are increased the hormone-sensitive lipase is phosphorylated and thereby activated (76). Evidence suggests a cyclic AMP-dependent protein kinase(s) mediates lipase activation. Intracellular increases in cyclic AMP activate the protein kinase(s). This activated protein kinase catalyzes the transfer of the gamma phosphate from ATP to the lipase and thereby activates it (4,49,82).

Corbin and Krebs (16) showed a cyclic AMP-dependent protein kinase was involved in the regulation of lipolysis when they described a cyclic AMP-dependent protein kinase in partially purified extracts from rat epididymal fat pads. The protein kinase exhibited a five-fold increase in histone phosphorylation in the presence of cyclic AMP. These partially purified extracts were also effective in phosphorylating casein and phosphorylase kinase.

Huttunen, Steinberg, and Mayer (38,39) showed that a cyclic AMP-

dependent protein kinase (phosphorylase b kinase kinase) from rabbit skeletal muscle catalyzed the ATP-dependent and cyclic AMP-dependent stimulation of lipolysis in rat adipose tissue extracts. Lipolysis was enhanced 44-93% in the 5.2P fraction. When the protein kinase or the cyclic nucleotide factor was absent activity was either significantly depressed or absent.

Huttunen and Steinberg (37) partially purified the lipase from rat epididymal fat pads 80-120 fold. Their work corroborated the results cited in previous experiments (16,38,39). Lipase activity was examined in four different purification fractions. In the first two steps of purification lipase activity was not dependent on added skeletal muscle protein kinase whereas in the third and fourth purification steps lipase activity was dependent on addition of protein kinase. This may indicate that endogenous protein kinase had been successfully removed in the third and fourth steps. The activation of the lipase paralleled its phosphorylation by ATP. These results suggested that the activation of adipose tissue lipase was mediated by cyclic AMP levels and that a protein kinase mediates this activation.

Corbin et al. (14) investigated the mechanism through which adenosine 3',5'-monophosphate regulated the protein kinase extracted from adipose tissue. The protein kinase was partially purified on a histone-Sepharose column and was stimulated 10-fold when cyclic AMP was added to the reaction mixture. The partially purified fractions phosphorylated histone, casein, skeletal muscle phosphorylase kinase and skeletal muscle glycogen synthetase in the

presence of cyclic AMP. Using casein-Sepharose column chromatography with cyclic AMP bound to the column, a cyclic AMP-independent fraction (catalytic subunit) was eluted. When rabbit muscle regulatory subunit (R) was added to this cyclic AMP-independent subunit in the absence of cyclic AMP activity was inhibited. However, if the regulatory subunit was added to the catalytic subunit in the presence of cyclic AMP activity was not inhibited.

Corbin et al. (17) attempted to characterize protein activity by measuring the degree of protein kinase activation in crude enzyme extracts from rat epididymal fat pads. Chromatography of the crude extract on Sephadex G-100 resolved two protein kinase peaks. One peak corresponded to the holoenzyme and was stimulated four-fold by cyclic AMP, while the second peak corresponded to the catalytic subunit and was cyclic AMP-independent. This research indicated that the protein kinase is composed of two subunits: a regulatory subunit which binds with the cyclic AMP and thereby releases the catalytic subunit from inhibition.

Soderling et al. (77) investigated the hormonal effects of epinephrine and insulin on cyclic AMP-dependent protein kinase activity in crude enzyme extracts from rat adipose tissue. Activity was measured as a protein kinase activity ratio (-cyclic AMP/+cyclic AMP). When epididymal fat pads were incubated with epinephrine the protein kinase activity ratio increased from 0.21 to 0.63. The increase in cyclic AMP induced by epinephrine corresponded to the measured increase in catalytic unit formation. Ten minute incubation with epinephrine and insulin produced a 70% inhibition in the protein

kinase activity ratio in homogenates. This effect was associated with a decrease in cyclic AMP and with the relative decrease in catalytic subunit formation. This data suggests that hormonal modification affects cyclic AMP concentrations which then alter protein kinase activity.

Zapf et al. (94) investigated cyclic AMP-dependent protein kinase activity in adipose tissue from streptozotocin-induced diabetic rats. Their results showed that labelled phosphate incorporation into casein in the absence or presence of cyclic AMP was linear for 7-10 minutes. Moreover, the protein kinase activity in the diabetic for both basal and cyclic AMP-stimulated activity was reduced 35% and cyclic AMP binding was decreased 30%. They attributed this decrease in protein kinase activity to depressed protein kinase synthesis due to hypo-insulinemia in the diabetic animals.

The db/db mutant of the C57BL/KsJ mouse exhibited reduced lipolytic activity in comparison to control animals. Kupiecki and Adams (46) investigated the phosphodiesterase, cyclic AMP-dependent protein kinase and adenylate cyclase systems in the young hyperinsulinemic, hyperglycemic db/db mouse for possible abnormalities which would explain this reduced lipolytic activity. Both high and low K_m phosphodiesterase activity in fat cell ghosts were similar in the db/db mouse and its control. Epinephrine and sodium fluoride (NaF) stimulated adenylyl cyclase activity in fat pad homogenates in the db/db mouse and controls to the same extent. In contrast, basal protein kinase activity was lower in the db/db mouse compared with its control while stimulated activity was greater in the db/db mouse

compared with its control. This suggested that protein kinase activity in the db/db mouse was more sensitive to cyclic nucleotide stimulation (46).

MATERIALS AND METHODS

Animals

Chinese hamsters were obtained from The Upjohn Company colony in Kalamazoo, Michigan. These animals came from highly inbred lines. Diabetic hamsters from the L line and nondiabetic hamsters from the M line were used in the experimentation. The hamsters ranged from three to nine months of age. All animals were individually housed in stainless-steel cages in a regulated environment maintained at 24°C. Animals were allowed water and food (Purina Mouse Breeder Chow, Ralston Purina Co., St. Louis, MO) ad libitum. Hamsters were classified as diabetic and nondiabetic on the basis of urine glucose as measured by the TesTape® (Eli Lilly and Co., Indianapolis, IN) method. Diabetics exhibited TesTape values of +3 and +4 while nondiabetics registered no TesTape values. Nondiabetics exhibited a range of nonfasting blood sugar values from 110 to 125 mg/dl whereas diabetics presented a range of nonfasting blood sugar values from 235 to 400 mg/dl (see Table 2).

The animals were weighed in a Mettler top loading pan balance prior to sacrifice. Urine was expressed from each animal at this time and was immediately tested with TesTape and Ketostix® (Ames Co., Elkhart, IN) for urine glucose and urine ketones, respectively. The animals were killed by orbital sinus exsanguination (64).

Blood Sugar and Plasma Insulin Measurement

Blood sugar and plasma insulin were measured for all the animals used in the comparison studies. Blood was collected via the orbital sinus in a chilled 12 x 75 mm disposable test tube. The test tube contained approximately 0.05 ml trasylol (Mobay Chemical Corp.) for every 1.0 ml blood collected.

Twenty microliters of whole blood was then placed in a Technicon Autoanalyzer vial containing 1.0 ml of 2.5% sodium fluoride. Blood glucose was quantitated using the Technicon Autoanalyzer (Technicon Instrument Company, Chauncey, NY) using a microglucose procedure (28) which was a modification of the method cited by Hoffman (36).

The remaining blood sample was centrifuged to separate the plasma. Plasma insulin was determined on 0.10 ml of thawed plasma. A radio-immunoassay method using a cellulose slurry to separate free insulin from bound insulin was used (92). A Packard Tri-Carb liquid scintillation spectrometer was used to count radioactivity.

Crude Enzyme Preparation

Immediately after the hamster had expired it was transferred to a clean surface and the abdominal, cranial and pleural cavities were exposed. The following tissues were extirpated: muscle from one hind leg, heart, epididymal fat pads, brain and liver. The liver was perfused by injecting chilled 0.01M sodium glycerol phosphate buffer with 0.001M EDTA, pH 6.5, into the hepatic portal vein prior to its removal. The total extraction time varied with

each animal but generally took 5-10 minutes. As the individual tissues were removed, they were placed into a beaker submerged in a dry ice-acetone bath and thus were quickly frozen. The tissues were left in the beaker for approximately 5-15 minutes after which time they were placed in individual labelled vials and stored in a freezer at -24°C .

The crude enzyme extracts were prepared by a procedure described by Kupiecki and Adams (46). The tissues were removed from the freezer and placed in ice to keep them chilled. The tissues were weighed individually and the weight was recorded. The tissue was then transferred to chilled Potter-Elvehjem all-glass homogenizers. Sodium glycerol phosphate buffer, 0.01M, with 0.001M EDTA, pH 6.5, was added in the appropriate volume:weight (v:w) ratio given in the following list: liver (5:1), heart (4:1), brain (3:1), epididymal fat pad (2:1), and skeletal muscle (3:1). These dilutions ensured a final protein concentration which could be accurately measured spectrophotometrically. The tissues were homogenized using a Vari-Speed stirrer (Precision Scientific Co., model #65730) using 5-10+ up and down strokes, depending on the tissue, at the lowest speed.

The individual homogenates were transferred to chilled centrifuge tubes. These tubes were placed in the chilled rotor head of a Damon IEC B20A centrifuge (Damon, IEC Division, Needham Hts., MS). The homogenates were centrifuged at 2°C for 15 minutes at $9000 \times g$. The supernatant was carefully removed without disturbing the pellet or the surface film using a Pasteur pipette. The individual super-

natants were again transferred to chilled centrifuge tubes and were centrifuged at 18,000 rpm at 2°C for 30 minutes (46). The crude enzyme extracts were then analyzed for protein concentrations spectrophotometrically (88).

Protein Kinase Assay

Protein kinase activity was determined using a modification of a method described by Corbin et al. (17) and Kupiecki and Adams (46). The reaction mixture contained 1.67×10^{-2} M potassium phosphate, pH 6.5, 5×10^{-4} M 1-methyl-3-isobutylxanthine, 10^{-2} M sodium fluoride, 5×10^{-4} M cyclic AMP (3',5'-adenosine cyclic phosphate, grade A, MW 329.2, Calbiochem), 1×10^{-4} M ATP (adenosine 5'-triphosphate, disodium salt from equine muscle, Sigma), 0.40 mg calf thymus histone (Sigma, Type IIA) and 0.080 mg protein in a volume of 0.22 ml. Radioactive ^{32}P -ATP was purchased as adenosine 5'-triphosphate, tetra-(triethylammonium) salt- γ - ^{32}P from New England Nuclear.

The reaction mixtures were maintained in ice until the reaction was initiated by the addition of ATP and enzyme extract. The reaction tubes were then incubated at 30°C for 16 minutes. Protein kinase activity was measured in triplicate.

The molar concentration of ATP was determined as described by Morell and Bock (58).

Chromatographic Analysis

The measurement of gamma-phosphate incorporation into the substrate followed a procedure described by Li and Felmlly (48). Twenty-five microliter

aliquots were withdrawn from the reaction mixtures at specified times during the incubation period and were pipetted onto Whatman 31 ET chromatography paper (Curtis Matheson Scientific, Inc., Elk Grove, IL). Gamma-phosphate incorporation into protein was measured by counting the radioactivity on the paper in 15 ml Bray's solution using a Packard Tri-Carb liquid scintillation spectrometer.

While this work was in progress, a simpler and more efficient method to measure gamma-phosphate incorporation into protein was reported by Witt and Roskoski (90). This method was used throughout the remaining experiments since comparable values were obtained using this procedure. With this method, twenty microliter aliquots from the reaction mixtures were pipetted onto cellulose phosphate paper (Whatman, P 81, Scientific Products) at specified intervals during the incubation period. Radioactivity on paper was counted immediately after successive washes with water, acetone and petroleum ether.

Statistical Analysis

Comparisons of protein kinase activity between tissues from non-diabetic and diabetic hamsters were calculated using a one-way analysis of variance program (75).

RESULTS

Preliminary studies were conducted on substrate affinity and ATP concentration using tissues from nondiabetic hamsters to establish conditions which have linear reaction kinetics and to provide information which allowed quantitation of protein kinase activity. The most effective substrate and ATP concentrations were then used in the comparison study of protein kinase activity in diabetic and nondiabetic hamsters.

The assumption was made that saturating concentrations of substrate were used based on evidence cited in the literature (16,47). Substrate specificity was examined using 0.40 mg protamine sulfate (Nutritional Biochem. Corp.), 0.40 mg histone (Sigma, Type IIA) and 0.60 mg casein (Sigma) in the final reaction mixture. Concentrations of 0.25×10^{-4} M ATP and 5×10^{-4} M cyclic AMP were used in these assays. The results of these studies are presented in Table 1.

Protein kinase extracts from skeletal muscle, heart muscle and epididymal fat pads consistently exhibited more phosphorylation with histone than with either casein or protamine sulfate. Phosphorylation of protamine sulfate was small when compared with histone phosphorylation for these three tissues in the two experiments. Heart muscle extracts phosphorylated casein to a small extent in comparison to histone. In one experiment epididymal fat pad and skeletal muscle extracts phosphorylated casein to a small extent while in another experiment casein phosphorylation was approximately one-half that observed with histone.

TABLE 1. Protein kinase activity measured with different substrates for five tissues from nondiabetic hamsters. Each number represents the mean of triplicate determinations with substrate and cyclic AMP over a period of four minutes

<u>TISSUE</u>	<u>EXPERIMENT NUMBER</u>	<u>PROTEIN KINASE ACTIVITY</u>		
		pmoles ^{32}P incorporated/mg protein <u>Histone</u>	<u>Casein</u>	<u>Protamine Sulfate</u>
Epididymal Fat Pad	1	2177	1067	923
	2	1309	222	349 ¹
Skeletal Muscle	1	2883	487	724
	2	1968	1222 ¹	-90
Heart Muscle	1	3195	399	524
	2	6533	373	-772
Liver	1	1273	424	786
	2	1079	1158 ¹	-95
Brain	1	2334	1435	5004
	2	2682	1793	5253

¹Number represents the mean of duplicate determinations

Greater substrate phosphorylation occurred with histone compared to protamine sulfate using liver enzyme extracts. When casein was used as the substrate equivocal results were obtained. In one experiment casein was phosphorylated to a greater extent than was histone while in another experiment casein was phosphorylated only one-third as much as histone.

Brain enzyme extracts exhibited the best substrate phosphorylation with protamine sulfate. Phosphorylation of protamine sulfate was twice as great as the phosphorylation of histone. Casein was phosphorylated approximately only half as much as histone.

Since extracts from at least three tissues exhibited better phosphorylation with histone than with the other two substrates, histone was used throughout the other preliminary studies and the comparison studies of protein kinase activity in diabetic and non-diabetic tissue extracts.

A preliminary study was done to examine the effect of two ATP concentrations (1.0×10^{-4} M and 0.25×10^{-4} M ATP) to assure linearity over a suitable time period since it was shown by Zapf *et al.* (89) that only a 1.0×10^{-3} M concentration produced linear phosphorylation for up to three minutes. Figure 1 through Figure 5 show results for protein kinase activity with two ATP concentrations using enzyme extracts from five tissues.

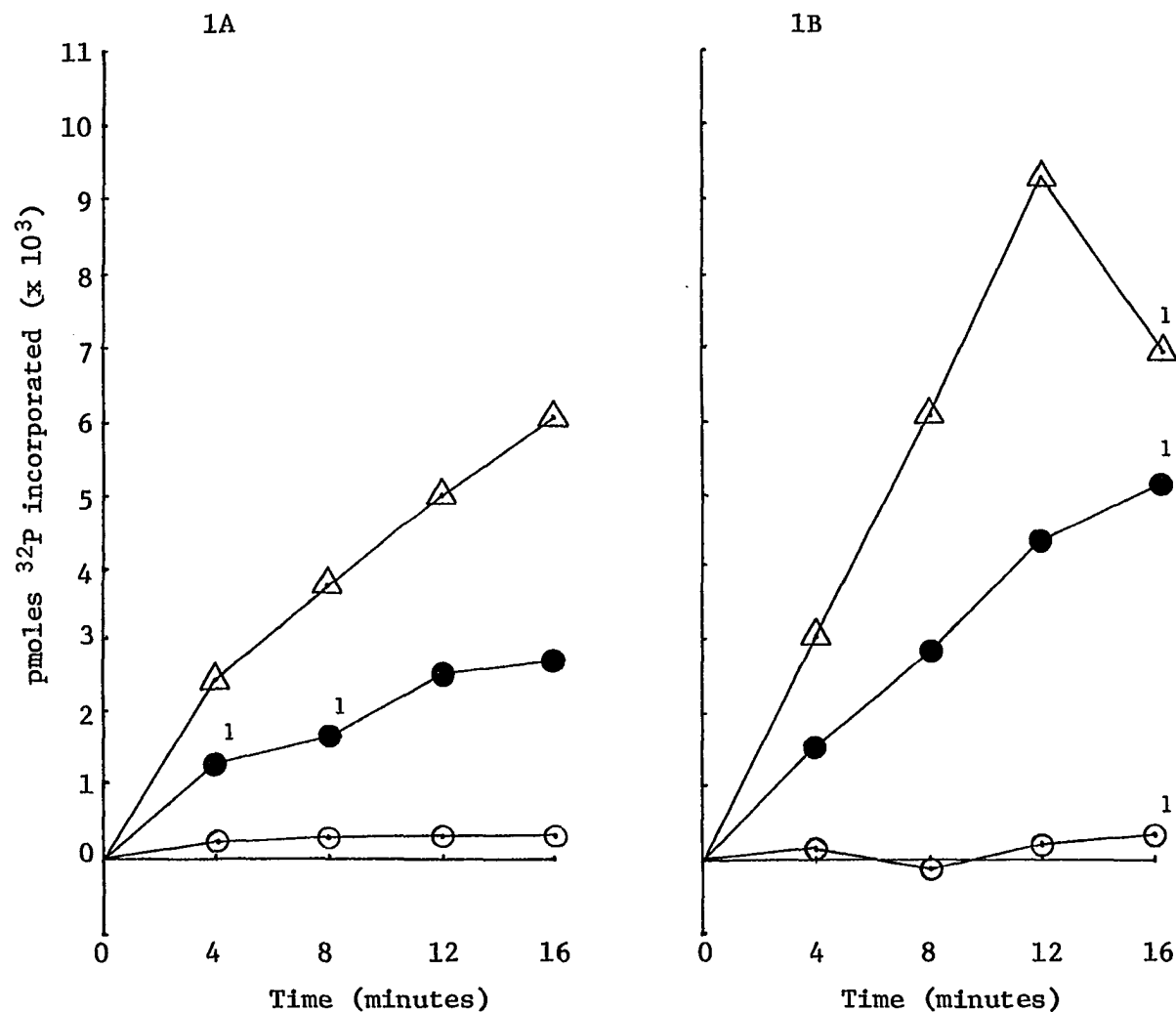
Protein kinase activity with brain extracts exhibited a greater degree of linearity for both stimulated and unstimulated histone phosphorylation over a 12 minute incubation period with 1.0×10^{-4} M ATP compared with the low ATP concentration (see Figure 1A and 1B).

Figures 2A and 2B show that both concentrations of ATP yield linear phosphorylation for 16 minutes using heart extracts. The curves are not strictly linear probably due to the small number of experiments used to calculate the mean. Figures 3A and 3B show the effects of high and low ATP concentrations on substrate phosphorylation using skeletal muscle enzyme extracts. Phosphorylation is linear throughout the 16 minute incubation period using 0.25×10^{-4} M ATP (see Figure 3A). Substrate phosphorylation using 1.0×10^{-4} M ATP is linear for 12 minutes and thereafter appears to diminish slightly (see Figure 3B). The number of experiments may account for this slight variance from linearity after the first 12 minutes. Figures 4A and 4B represent substrate phosphorylation using enzyme homogenates from liver tissue. Basal and cyclic AMP-stimulated histone phosphorylation was linear throughout the 16 minute incubation period with high ATP and low ATP concentrations. Substrate phosphorylation using epididymal fat pad homogenates with high and low ATP concentrations is shown in Figures 5A and 5B. Again both ATP concentrations gave linear phosphorylation allowing for the limited number of experiments.

Table 2 lists pertinent characteristics of nondiabetic and diabetic hamsters used in the comparison studies of protein kinase activity in different tissues. The characteristics of the individual hamsters are listed in the designated column and a mean and standard error are given when appropriate.

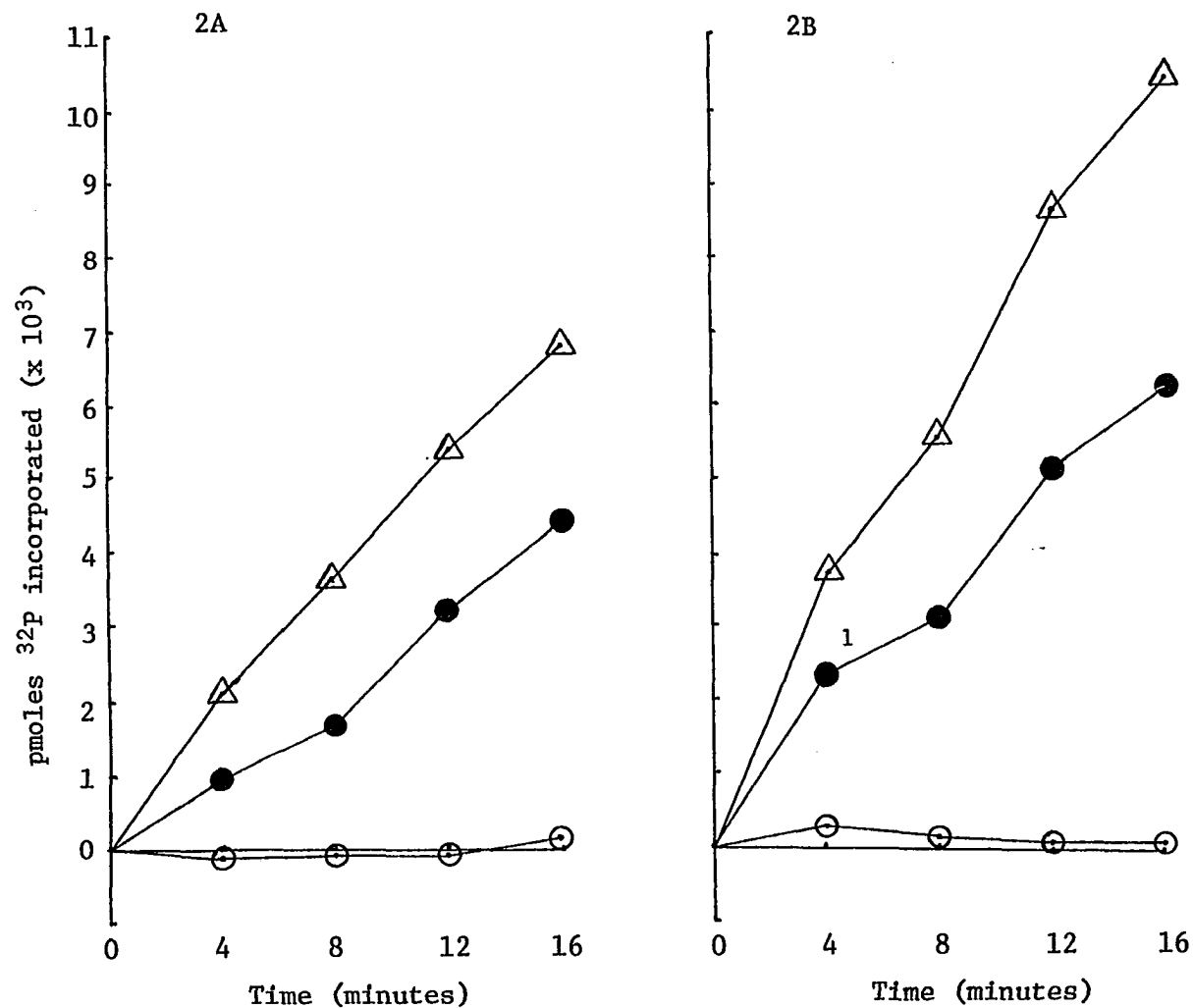
Urine TestTape values for diabetics prior to experimentation were +3 and +4 while the nondiabetics registered +0 values for urine glucose. Urine ketones measured with Ketostix (not listed) were negative for

Figure 1. A comparison of the effect of ATP concentrations on the reaction rate in the protein kinase assay using brain homogenates. Figure 1A and Figure 1B show the effect of 0.25×10^{-3} M ATP and 1×10^{-3} M ATP, respectively. The effect was examined in reaction mixtures without substrate (\odot — \odot), with histone (\bullet — \bullet) and with histone and cyclic AMP (\triangle — \triangle). The means of three experiments using low ATP concentrations and three experiments using high ATP are plotted at each time interval unless otherwise indicated.



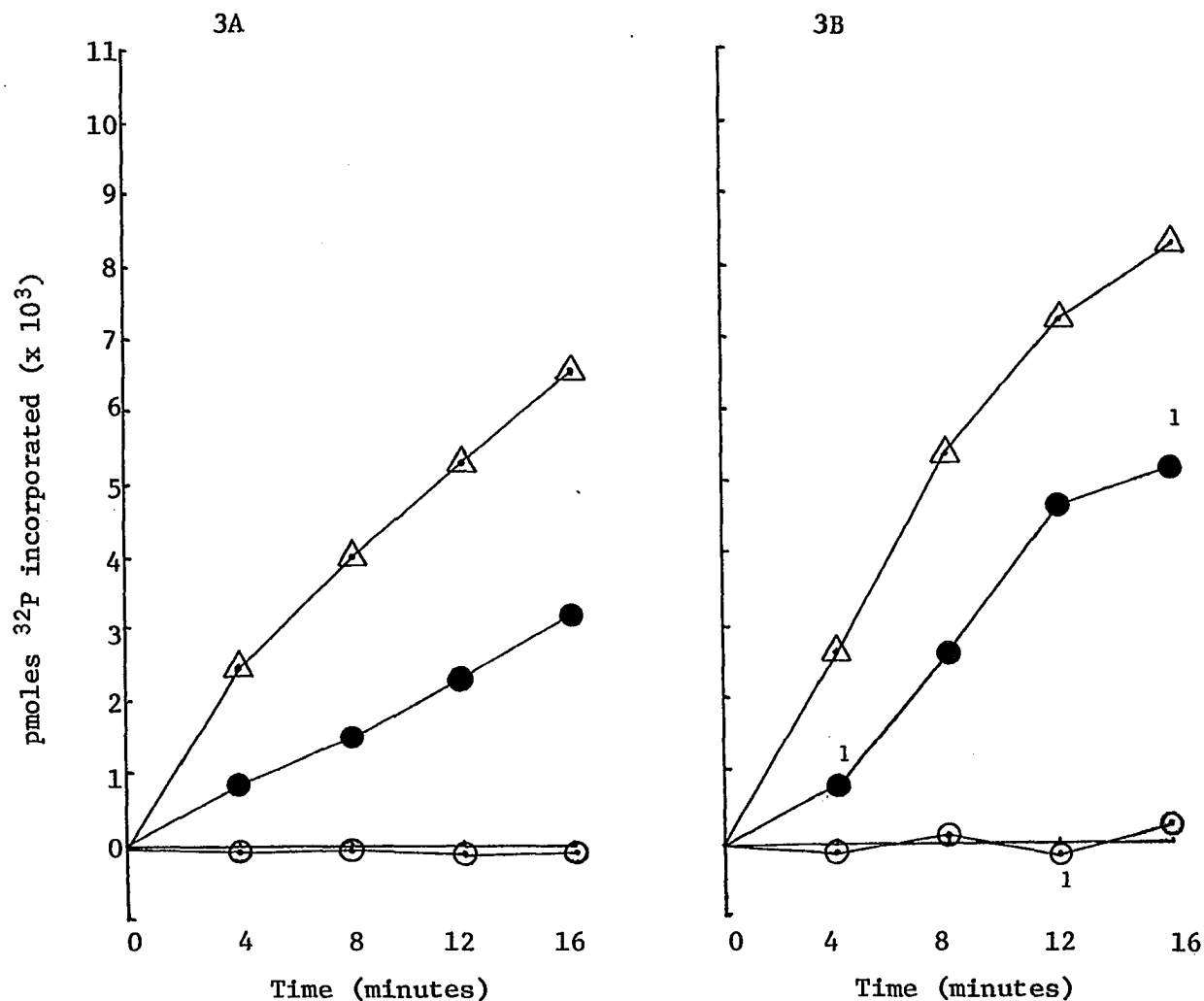
¹Mean calculated from two values recorded at that time interval

Figure 2. A comparison of the effect of ATP concentrations on the reaction rate in the protein kinase assay using heart homogenates. Figure 2A and Figure 2B show the effect of 0.25×10^{-3} M ATP and 1×10^{-3} M ATP, respectively. This effect was examined in reaction mixture without substrate (\bigcirc — \bigcirc), with histone (\bullet — \bullet) and with histone and cyclic AMP (\triangle — \triangle). The means of three experiments using low ATP concentrations and three experiments using high ATP are plotted at each time interval unless otherwise indicated.



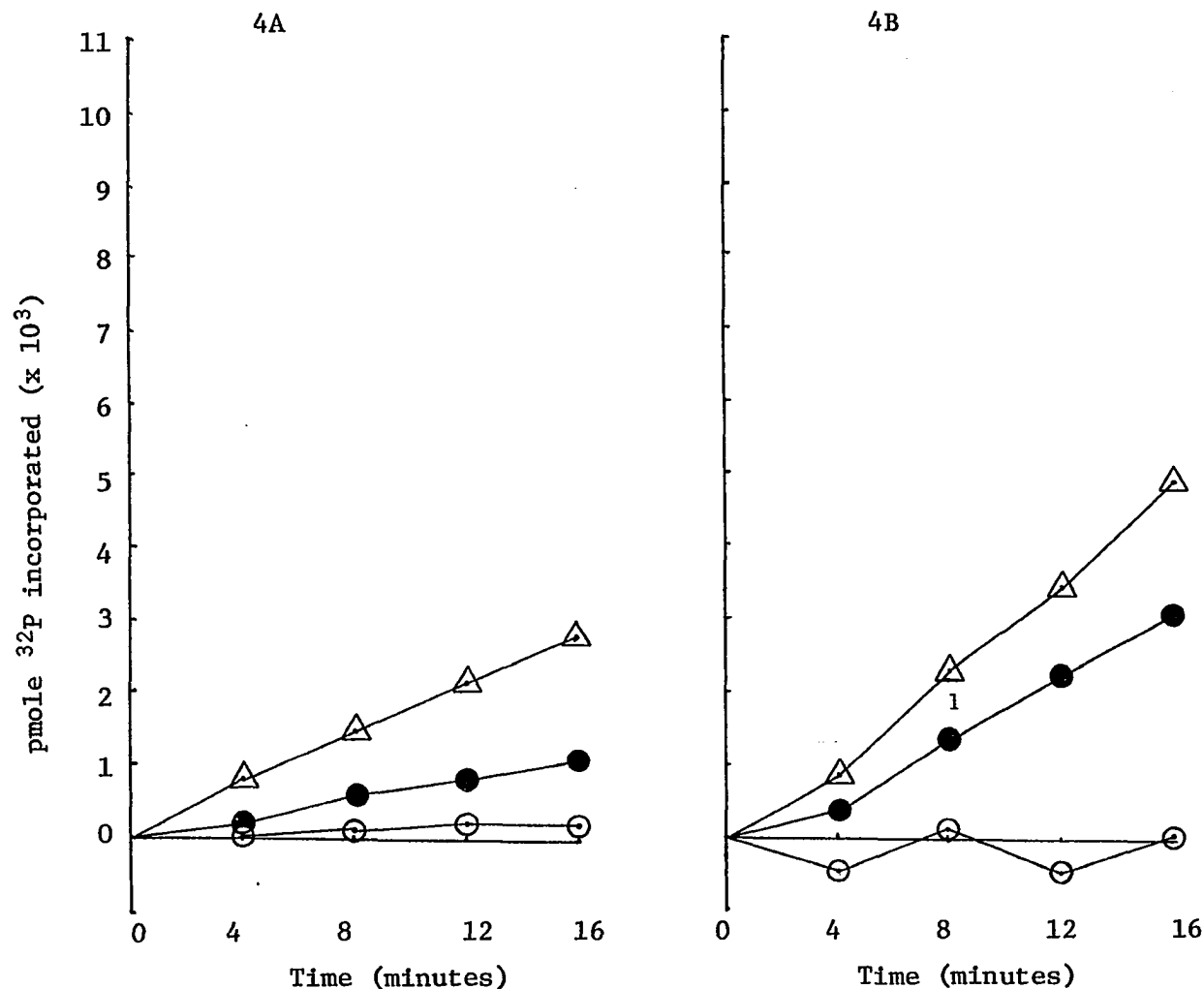
¹Mean calculated from two values recorded at that time interval

Figure 3. A comparison of the effect of ATP concentrations on the reaction rate in the protein kinase assay using skeletal muscle homogenates. Figure 3A and Figure 3B show the effect of 0.25×10^{-3} M ATP and 1×10^{-3} M ATP, respectively. This effect was examined in reaction mixtures without substrate (\bigcirc — \bigcirc), with histone (\bullet — \bullet) and with histone and cyclic AMP (\triangle — \triangle). The means of three experiments using low ATP concentrations and four experiments using high ATP are plotted at each time interval unless otherwise indicated.



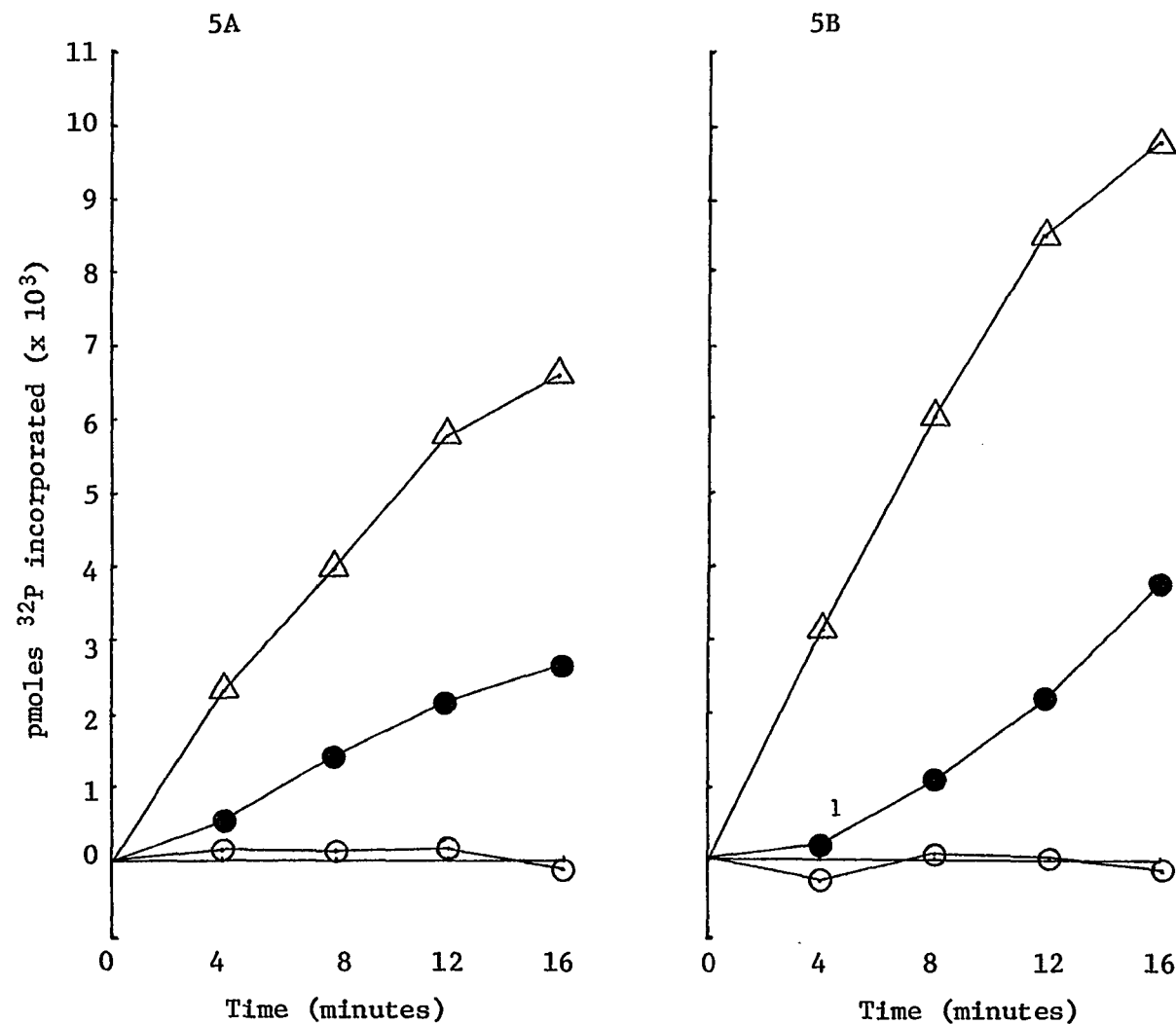
¹Mean calculated from three values recorded at that time interval.

Figure 4. A comparison of the effect of ATP concentrations on the reaction rate in the protein kinase assay using liver homogenates. Figure 4A and Figure 4B show the effect of 0.25×10^{-3} M ATP and 1×10^{-3} M ATP, respectively. This effect was examined in reaction mixtures without substrate (\bigcirc — \bigcirc), with histone (\bullet — \bullet) and with histone and cyclic AMP (\triangle — \triangle). The means of three experiments using low ATP concentrations and four experiments using high ATP are plotted at each time interval unless otherwise indicated.



¹Mean calculated from two values recorded at that time interval

Figure 5. A comparison of the effect of ATP concentrations on the reaction rate in the protein kinase assay using epididymal fat pad homogenates. Figure 5A and Figure 5B show the effect of 0.25×10^{-3} M ATP and 1×10^{-3} M ATP, respectively. This effect was examined in reaction mixtures without substrate (\bigcirc — \bigcirc), with histone (\bullet — \bullet) and with histone and cyclic AMP (\triangle — \triangle). The means of three experiments using low ATP concentrations and three experiments using high ATP are plotted at each time interval unless otherwise indicated.



¹Mean calculated from two values recorded at that time interval

Table 2. Characteristics of diabetic and nondiabetic Chinese hamsters used in comparison studies of protein kinase activity in crude enzyme preparations

<u>LINE AND NUMBER</u>	<u>SEX</u>	<u>TESTAPE VALUE</u>	<u>AGE AT SACRIFICE (days)</u>	<u>NONFASTED BODY WEIGHT (grams)</u>	<u>NONFASTED BLOOD GLUCOSE (mg/dl)</u>	<u>NONFASTED PLASMA INSULIN (μU/1.0 ml plasma)</u>
Nondiabetic						
M12-90	F	+0	420	24.46	114	55
M00180	M	+0	278	31.49	120	63
M13-58	M	+0	367	34.77	125	90
M00176	M	+0	275	33.96	110	78
M00190	M	+0	263	29.52	125	52
M13-48	F	+0	424	29.11	112	54
MEAN \pm STANDARD ERROR ¹				30.55 \pm 1.53	120 \pm 3.5	65.3 \pm 6.2
Diabetic						
L28-60	M	+4	234	33.25	327	72
L00118	M	+3	283	42.46	235	96
L28-38	M	+4	241	36.31	400	29
L28-59	M	+4	249	35.17	345	57
L00125	M	+4	262	35.66	260	135
L28-66	M	+3	248	38.32	235	155
MEAN \pm STANDARD ERROR				36.94 \pm 1.30 ²	310.3 \pm 24.4 ³	90.6 \pm 19.5 ⁴

¹ The mean \pm standard error was calculated for nonfasted body weight, nonfasted blood glucose, and nonfasted plasma insulin for each experimental group.

² p <0.010 P value vs. nondiabetic

³ p <0.000 P value vs. nondiabetic

⁴ p <0.245 P value vs. nondiabetic

both groups.

A significant difference ($p < 0.001$) in the mean blood glucose level between the two groups was measured. The mean nonfasted blood glucose for diabetic and nondiabetics was 310 mg/dl and 120 mg/dl, respectively.

The mean nonfasted plasma insulin measured in $\mu\text{U/ml}$ was not significantly different between the diabetic and nondiabetic groups. A wide range of values was observed in both groups. Plasma insulin measurement for nondiabetics ranged from 52 to 90 $\mu\text{U/ml}$ and for diabetics ranged from 29 to 155 $\mu\text{U/ml}$. The mean and standard error for nondiabetics and diabetics was $65.3 \pm 6.2 \mu\text{U/ml}$ and $90.6 \pm 19.5 \mu\text{U/ml}$, respectively.

The results of the comparison studies on protein kinase activity in five tissues from diabetic and nondiabetic hamsters are graphed in Figure 6 through Figure 10. In these figures results are expressed as picomoles of phosphate incorporated into substrate over a sixteen minute incubation period. The data shows only basal and cyclic AMP-stimulated activity because phosphorylation of endogenous protein was not evident (see Figure 1 through Figure 5). A comparison of basal protein kinase activity for each tissue from diabetic and nondiabetic hamsters using a one-way analysis of variance showed no significant difference for basal activity between diabetics and nondiabetics. Moreover, no significant difference in cyclic AMP-stimulated phosphorylation of histone was exhibited between diabetics and nondiabetics.

Figure 6. Protein kinase activity in tissue homogenates of brain. Figure 6A shows protein kinase activity of nondiabetic hamsters and Figure 6B shows protein kinase activity of diabetic hamsters with basal (●—●) and cyclic AMP-stimulated (○—○) activity plotted for both animals. The means and standard errors are plotted at each time interval. The number in parentheses denotes the number of animals used. Neither unstimulated ($p < 0.60$) and stimulated ($p < 0.78$) protein kinase activity was significantly different between experimental groups.

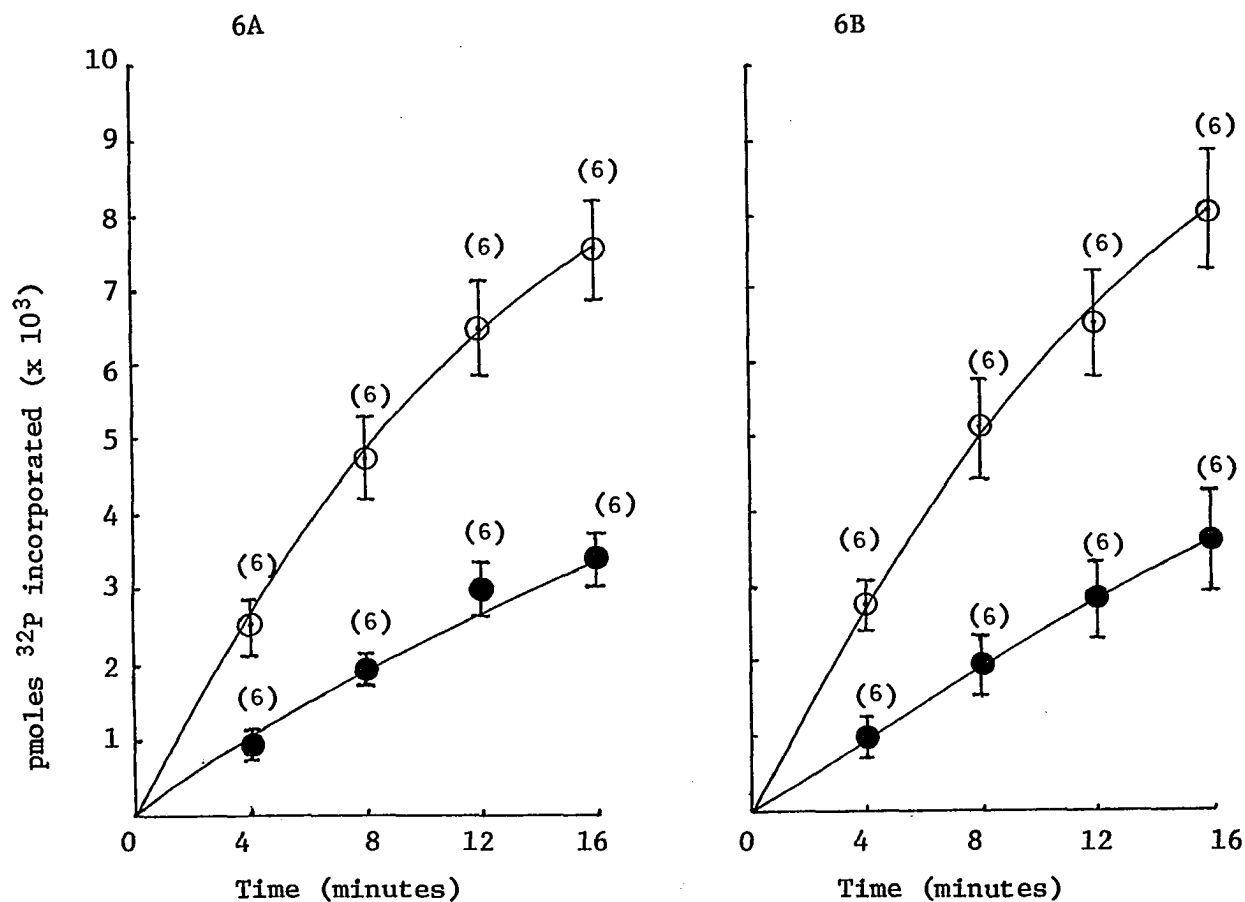


Figure 7. Protein kinase activity in tissue homogenates of heart muscle. Figure 7A shows protein kinase activity of nondiabetic hamsters and Figure 7B shows protein kinase activity of diabetic hamsters with basal (●—●) and cyclic AMP-stimulated (○—○) activity plotted for both animals. The means and standard errors are plotted at each time interval. The number in parentheses denotes the number of animals used. Neither unstimulated ($p < 0.35$) and stimulated ($p < 0.10$) protein kinase activity was significantly different between experimental groups.

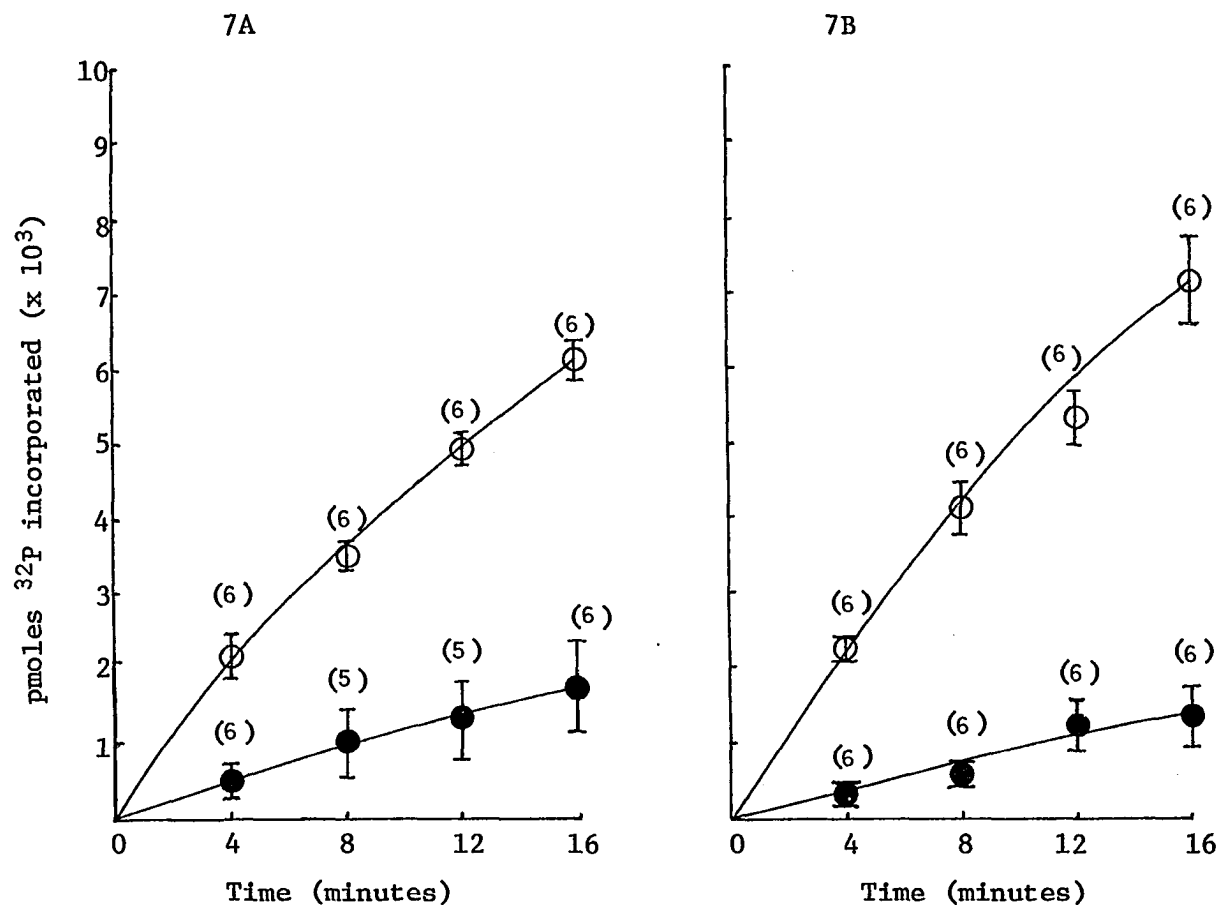


Figure 8. Protein kinase activity in tissue homogenates of epididymal fat pads. Figure 8A shows protein kinase activity on nondiabetic hamsters and Figure 8B shows protein kinase activity of nondiabetic hamsters with basal (●—●) and cyclic AMP-stimulated (○—○) activity plotted for both animals. The means and standard errors are plotted at each time interval. The number in parentheses denotes the number of animals used. Neither unstimulated ($p < 0.21$) and stimulated ($p < 0.22$) protein kinase activity was significantly different between experimental groups.

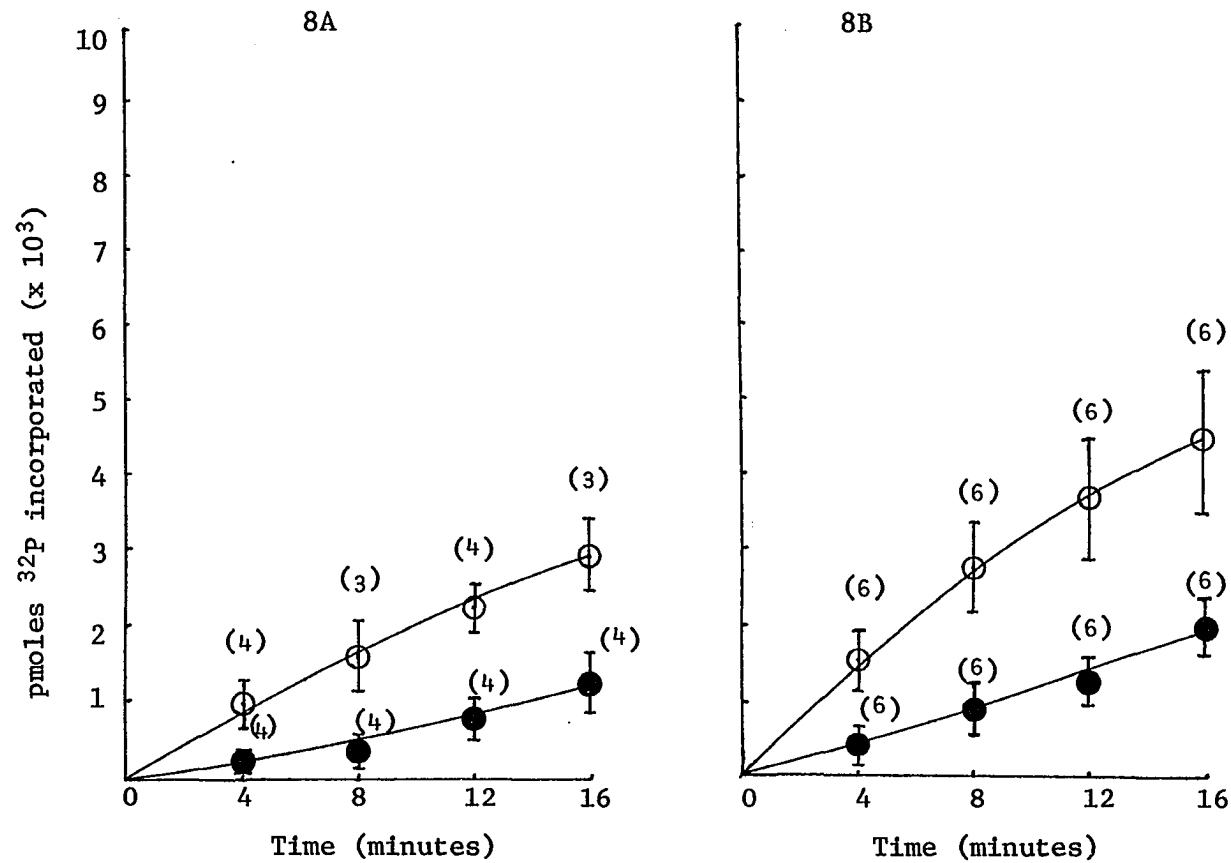


Figure 9. Protein kinase activity in tissue homogenates of liver. Figure 9A shows protein kinase activity of nondiabetic hamsters and Figure 9B shows protein kinase activity of diabetic hamsters with basal (●—●) and cyclic AMP-stimulated (⊙—⊙) activity plotted for both animals. The means and standard errors are plotted at each time interval. The number in parentheses denotes the number of animals used. Neither unstimulated ($p < 0.55$) and stimulated ($p < 0.57$) protein kinase activity was significantly different between experimental groups.

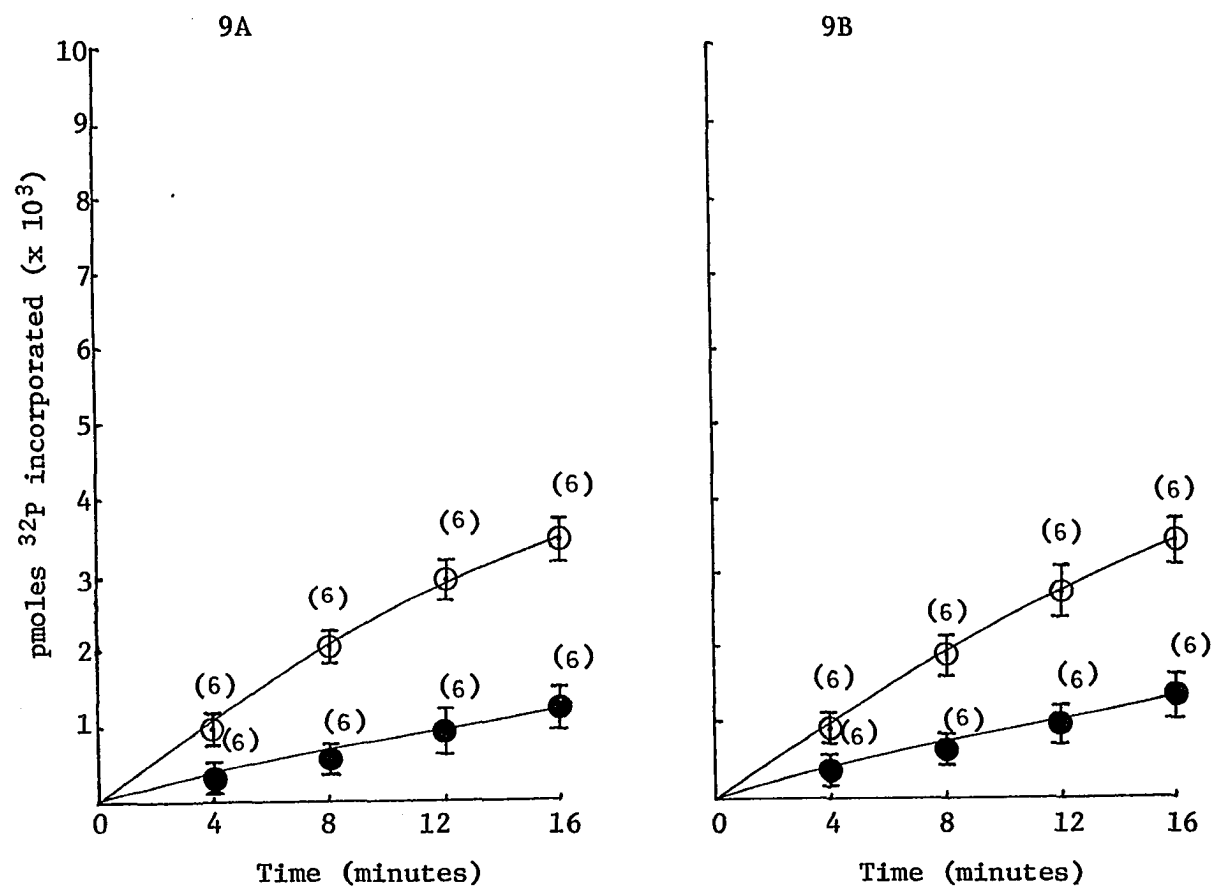
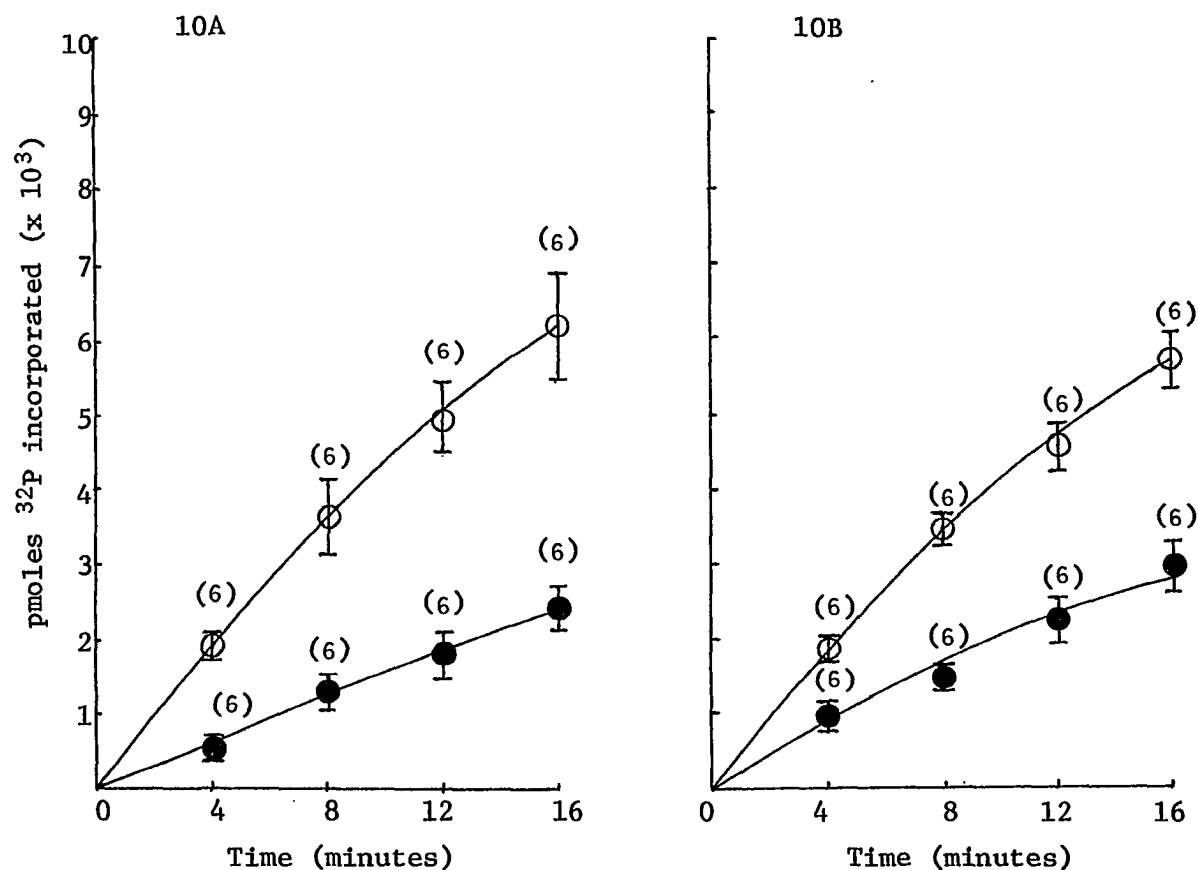


Figure 10. Protein kinase activity in tissue homogenates of skeletal muscle. Figure 10A shows protein kinase activity of nondiabetic hamsters and Figure 10B shows protein kinase activity of diabetic hamsters with basal (●—●) and cyclic AMP-stimulated (○—○) activity plotted for both animals. The means and standard errors are plotted at each time interval. The number in parentheses denotes the number of animals used. Neither unstimulated ($p < 0.16$) and stimulated ($p < 0.53$) protein kinase activity was significantly different between experimental groups.



DISCUSSION

Protein kinase(s) regulate cellular activities through the phosphorylation of proteins such as enzymes (68). In choosing a suitable substrate with which to measure protein kinase activity we make the assumption that the substrate phosphorylation reflects the intracellular protein kinase activity in tissues. Therefore preliminary experiments were conducted on three substrates to determine which one would exhibit adequate phosphorylation for all five tissues.

Three proteins are commonly used as substrates for protein kinase: histone, casein, and protamine sulfate. In an examination of substrate affinity with epididymal fat pad homogenates from the rat, Corbin and Krebs (16) reported that greater phosphorylation occurred with histone than with casein. In another paper, casein was reported as a suitable substrate in partially purified fat pad homogenates from rats (94). Walsh and his associates (87) indicated that histone was phosphorylated to a greater degree than protamine sulfate by rabbit skeletal muscle extracts. Casein, protamine sulfate, and histone have been reported as suitable substrates for measuring protein kinase activity under various assay conditions with bovine heart muscle (3, 65). Langan (47) reported that calf liver homogenates phosphorylated histone while little phosphorylation was observed with protamine sulfate. With brain extracts from rabbit, Miyamoto et al. (57) showed protamine sulfate was phosphorylated to a greater extent than histone. These results suggest that protein kinases from different

tissues have specificities for the exogenous non-enzyme substrates they phosphorylate. This is not surprising since the endogenous substrates are likely to vary from tissue to tissue.

The results of experiments reported in this thesis indicated that skeletal muscle, heart muscle, liver and epididymal fat pad extracts from the Chinese hamster phosphorylated histone to a greater extent than the other two substrates. Casein appeared to be a suitable substrate for liver extracts from the Chinese hamster. This suggests that histone was structurally similar to the endogenous substrate in skeletal muscle, heart muscle and epididymal fat pads and consequently better phosphorylated than was casein or protamine sulfate. These results are similar to those cited in the literature and discussed above which indicated that histone was phosphorylated to a greater extent in epididymal fat pads, skeletal muscle, and liver from different animals. In contrast, protamine sulfate served as the best substrate for brain homogenates from the Chinese hamster. Rabbit brain homogenates showed the same specificity for protamine sulfate (57). This suggests that protamine sulfate is more similar to the in vivo substrate for brain than either histone or casein. For the convenience of assaying for protein kinase activity in five tissues of the Chinese hamster, histone was used throughout the comparison studies despite the fact that protamine sulfate was phosphorylated better by brain homogenates.

In regard to the ATP concentrations, Zapf and his coworkers achieved linearity of substrate phosphorylation using 10^{-5} M cyclic AMP and 1×10^{-3} M ATP for 7-10 minutes in epididymal fat pad ex-

tracts from diabetic and nondiabetic rats. But when concentrations lower than 1 mM (2×10^{-4} and 5×10^{-5} M) ATP were used the reaction rate became nonlinear after 2-3 minutes (94). The results from experiments reported in this paper (Figure 1 through Figure 5) indicate that 0.25×10^{-4} mM and 1×10^{-4} M ATP concentrations provide relative linearity for at least 12 minutes for protein kinase activity in tissues from diabetic and nondiabetic hamsters. Therefore, the higher ATP concentration was used in the comparison studies of protein kinase activity in tissues from diabetic and nondiabetic hamsters.

The comparison studies of protein kinase activity (Figure 6 through Figure 10) indicate that there was no significant difference between diabetic and nondiabetic basal and cyclic AMP-stimulated protein kinase activity in the five tissues examined from the Chinese hamster. In this respect, the Chinese hamster is unlike other diabetic animals. This was surprising since protein kinase activity in brain, skeletal muscle, heart muscle, epididymal fat pad and liver extracts from the diabetic and nondiabetic Chinese hamster was expected to differ since research cited in the literature has shown a difference in protein kinase activity from tissues in the spontaneously diabetic db/db mouse and in the chemically-induced diabetic animals and their controls as cited in the introduction. However, if insulin is important in regulating protein kinase activity, these results are not surprising since plasma insulin levels were not different in the diabetic and nondiabetic Chinese hamster. A further difference between the Chinese hamster and all other diabetic animals studied in the literature, with the exception of the db/db mouse, was

that the Chinese hamster was spontaneously diabetic. Therefore, it is suggested that there appears to be no consistent relationship between protein kinase activity and diabetes in diabetic animals and consequently it may not play an important role in the regulation of blood sugar in diabetes.

SUMMARY

1. The protein kinase activity was compared in five different tissue extracts of the diabetic and nondiabetic Chinese hamster.
2. Protein kinase activity was determined using a modification of a method described by Corbin et al. (17) and Kupiecki and Adams (46).
3. Preliminary studies were conducted on substrate affinity and ATP concentration using tissue extracts from nondiabetic hamsters to establish conditions which have linear kinetics and to provide information which allowed quantitation of protein kinase activity.
4. Brain enzyme extracts exhibited the best substrate phosphorylation with protamine sulfate. Skeletal muscle, heart muscle, and epididymal fat pads consistently exhibited more phosphorylation with histone than with either casein or protamine sulfate. Liver extracts phosphorylated histone and casein.
5. Linearity over a suitable time period was obtained with 0.25×10^{-4} M and 1.0×10^{-4} M ATP for the five tissue extracts from nondiabetic and diabetic Chinese hamsters.
6. Plasma insulin levels and nonfasted body weight were not significantly different between diabetic and control animals whereas nonfasted blood glucose was significantly different between diabetic and nondiabetic animals.
7. A comparison of protein kinase activity of five tissue extracts from diabetic and nondiabetic hamsters revealed no significant differences in basal activity. Cyclic AMP-stimulated protein kinase activity of the diabetic and nondiabetic Chinese hamsters was not significantly different in five tissue extracts.

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