ALTERATIONS IN SUBCELLULAR ORGANELLES OF THE HEART DURING CHRONIC DIABETES

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ALTERATIONS IN SUBCELLULAR ORGANELLES OF

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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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ABSTRACT

Chronic diabetes in animals and humans is known to be accompanied by depressed cardiac performance but the subcellular mechanisms which may be responsible for this cardiomyopathy are largely unknown. Therefore, the purpose of this investigation was to study the function and composition of three subcellular organelles in the heart, the myofibrils, sarcolemma and mitochondria, in order to obtain some information regarding the extent of their participation in the cardiomyopathy.

Diabetes was induced in male Sprague-Dawley rats (200-250 g) by a single intravenous injection of streptozotocin at a dosage of 65 mg/kg body weight. Hearts were removed from the animals 8 weeks later and ventricular tissue processed for the isolation of myofibrils, sarcolemmal or mitochondrial fractions by differential centrifugation.

The Mg^{2+}- and Ca^{2+}-stimulated ATPase activities of myofibrils from diabetic hearts were significantly lower than control values. Diabetic animals injected with approximately 2U/day insulin exhibited myofibrillar ATPase activities which were similar to control. Although Ca^{2+}-stimulated ATPase activity was depressed in diabetic myocardium, the dependency of diabetic myofibrils on free calcium concentration was not different from that of control. The basal and Ca^{2+}-stimulated ATPase activities in diabetic rats demonstrated a greater sensitivity to KCl than control preparations. The myofibrillar basal ATPase, unlike Ca^{2+}-stimulated ATPase, in diabetic animals exhibited an altered sensitivity to ethylene glycol and N-ethylmaleimide modification. Myofibrillar sulfhydryl reactivity was depressed in samples from diabetic animals in comparison to control and insulin-treated diabetic animals. These results would suggest the presence of subtle structural and conformational changes in myofibrils from diabetic rat heart.

Heart sarcolemmal membranes were isolated by the hypotonic shock-LiBr treatment. Chronic streptozotocin-induced diabetes in rats was observed to be associated with a significant loss in the ability of isolated cardiac sarcolemmal membranes to bind calcium at both high and low Ca^{2+} concentrations. This effect was insulin-reversible. The sialic acid residues, which are considered to represent a superficial calcium pool in sarcolemma, were depressed in content in preparations from diabetic rats. Insulin treatment of the diabetic rats normalized sarcolemmal sialic acid content. The depression in sarcolemmal sialic acid content in diabetic preparations could not be accounted for by any alteration in membrane sialyltransferase activity. Neuraminidase treatment reduced sarcolemmal calcium binding by 37% in control preparations but had no effect on diabetic preparations. Acidic phospholipids such as phosphatidylinositol and phosphatidylserine, which have the ability to bind calcium in isolated sarcolemmal membranes, were not altered during diabetes. However, an increase in lysophosphatidylcholine and decreases in phosphatidylethanolamine and diphosphatidylglycerol contents were observed in membranes isolated from diabetic rats. Cholesterol content of diabetic rat cardiac sarcolemma was also increased. Ca^{2+}-dependent ATPase activity, which may represent another parameter for the interaction of calcium with sarcolemma, was elevated in diabetic preparations. This increase in enzyme activity was associated with alterations in the Vmax values without any changes in the Km and Ka values.

Sarcolemmal Mg^{2+}-dependent ATPase activity was elevated whereas 5'-nucleotidase and K^{+}-pNPPase activities in diabetic rat hearts were depressed in comparison to control preparations. Although patent Na^{+}, K^{+}-ATPase and patent ouabain-sensitive Na^{+}, K^{+}-ATPase activities were

unaltered, latent Na^+, K^+-ATPase activities, as determined in membranes after alamethicin or deoxycholate treatments, were found to be significantly depressed in diabetic animals. A depression in the latent Na^+, K^+-ATPase activity in diabetic preparations was also observed in membranes prepared by the sucrose density gradient method. Insulin-treated diabetic rats were observed to have normalized latent Na^+, K^+-ATPase activities. In order to determine the role which cholesterol may play in the defect in Na^+, K^+-ATPase activity during diabetes, membranes were treated with filipin, an agent known to bind with cholesterol residues. Sarcolemmal Na^+, K^+-ATPase activity in diabetic heart was more resistant to treatments with filipin.

Oxidative phosphorylation capacity and Ca^{2+} accumulation of isolated mitochondria from diabetic rat heart was also studied. The state 3 respiration, respiratory control index, oxidative phosphorylation rate and Mg^{2+}-dependent ATPase activities were depressed in mitochondria from diabetic hearts and these changes were partially reversible upon 2 weeks of insulin therapy. Mitochondrial calcium uptake, but not calcium binding, was also decreased in diabetes but this change was fully reversible by insulin administration. The observed alterations in mitochondrial function could not be explained on the basis of any changes in mitochondrial lipid and protein composition.

The results of this study provide some information regarding the subcellular loci of the defect which may be responsible in part for the cardiomyopathy in diabetes. The depression in myofibrillar ATPase activity may closely relate to the cardiodepression observed in chronically diabetic rats. These results also suggest that chronic experimental diabetes is associated with defects in sarcolemmal enzymatic

activities and composition which may have important implications with regard to the maintenance of ionic homeostasis in the myocardium. The generalized depression in mitochondrial function may influence cardiac metabolism and also limit performance of the heart. These subcellular defects appear closely associated with the condition of chronic insulin deficiency since most of the lesions were reversible upon administration of insulin to the diabetic animals.

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I. INTRODUCTION AND STATEMENT OF THE PROBLEM

In a historical sense, diabetes mellitus has probably been a remarkably close and insidiously lethal compatriot of man since the beginnings of civilization. Crude descriptions of the disease have been found amongst ancient Egyptian relics and in Indian writings long before the birth of Christ (Marble, 1971). Unfortunately, these observations of antiquity were not dramatically improved upon until the 18th century when Dobson (1776) linked the disease to a sugar abnormality and Cawley (1788) suggested that the pancreas may be intimately involved in the sickness. It was over 100 years before Cawley's theory was proven correct by Von Mering and Minkowski (Marble, 1971) and, with Banting and Best's famous discovery of insulin in 1921 diabetes mellitus was thought to be "cured". However, although the post-insulin era has spawned a tremendous quantity of research on education, pharmacology, pathogenesis and prophylactic treatment of the disease, it remains an unescapable fact that diabetes mellitus is at best treatable but certainly not curable. Indeed, although the average life expectancy of a diabetic patient has increased dramatically in the post-insulin era (Christlieb, 1973), diabetes mellitus still represents a significant health problem to the diabetic population.

In the early 1940's there were a little over ½ a million reported cases of clinical diabetes in the U.S. (Rynearson and Hildebrand, 1941). By 1965 there were 2½ million cases (Marks et al, 1971), by 1973 there were 5 million (Fact Sheet on Diabetes, 1973) which further increased until at present there are estimated to be 10 million Americans showing overt symptoms of diabetes mellitus (Notkins, 1979; Davidson, 1981). This may merely represent the tip of the iceberg. It is estimated that so many cases of diabetes go unreported or undiagnosed that as many as

20 million people in the U.S. may be diabetic (Davidson, 1981). The average American citizen today will have a 1 in 5 chance of developing diabetes during his or her lifetime (Notkins, 1979).

However, the crucial statistical factor which must ultimately determine the significance of any disease to the human race lies in its mortality figures. In 1900, diabetes mellitus was ranked as 27th on the list of causes of death in the U.S. (Tokuhata et al, 1975). This infamous ranking has risen alarmingly to the point today where, behind cardiovascular disease and cancer, diabetes mellitus has been reported to be the third leading cause of death in the U.S. (Notkins, 1979).

The factors responsible for these abnormally high mortality characteristics are probably numerous, but much can be learned from statistics accumulated on the cause of death in diabetic patients. Failure of the cardiovascular system is the leading cause of death in the diabetic population (Goodkin, 1974). However, such was not always the case. Data from the pre-insulin era indicated 20% of deaths in diabetic patients were due to cardiac failure, 8% due to coronary vessel disease and 14% due to diabetic coma (Entmacher et al, 1964). Forty years later, figures of 51% for cardiac failure, 28% for coronary vessel disease and 1% due to diabetic coma have been reported as causes of death in diabetic patients (Entmacher et al, 1964). It is obvious, therefore, that the nature of the disease has become dramatically altered in the wake of the advent of insulin treatment. Insulin administration has significantly extended the life of the patient and virtually eliminated comatosis as a cause of death but it has also aggravated arteriosclerotic heart disease and other features of myocardial degeneration. Death due to diabetes is now a clinical problem of cardiovascular dysfunction. Its manifestation in these patients is dependent upon and secondary to the presence of a diabetic

condition in the patient.

Extensive epidemiological work has revealed much concerning the characteristics of the problem of cardiovascular disease during diabetes. The presence of diabetes will increase the chance of incurring an important cardiovascular event by 2 fold in the male population and 3-5 fold in the female population (Kannel and McGee, 1979). Diabetes can increase the possibility of suffering a myocardial infarction by 2.5 -5 times the normal risk (Sievers et al, 1961; Pell and D'Alonzo, 1963). Congestive heart failure and shock were more prevalent during the acute myocardial infarction (Partamian and Bradley, 1965). Mortality rate for diabetics after a heart attack is higher than the non-diabetic population (Mintz and Katz, 1974; Tansey et al, 1977; Soler et al, 1975).

Numerous factors may be responsible for these alarming statistics, however, in the final analysis three major factors appear to largely account for the increased incidence of cardiac injury during diabetes. These three factors may be implicated in varying extents, in concert or acting independently, in both human and experimental diabetic models. They are: 1) Major vessel disease in the form of atherosclerosis; 2) Microvascular alterations in the heart; 3) The presence of a primary myopathic disorder in the cardiac muscle.

There appears to be little doubt that the average diabetic patient is more susceptible to significant atherosclerotic development in the macrovasculature of the heart in comparison to the general population. Numerous studies have documented enhanced coronary artery occlusive disease in the diabetic myocardium (Stearns et al, 1947; Clawson and Bell, 1949; Liebow et al, 1955). This problem exists in the peripheral vasculature as well, resulting in retinopathy, nephropathy and various

other serious circulatory lesions (e.g. gangrene). Unfortunately, the significance and the conclusions of many of these early investigations were severely limited by many flaws in the experimental protocol. Firstly, determination of atherosclerotic development was quantified in a largely subjective manner. Secondly, there was little or no attempt made to correlate the process with the duration or severity of the disease. Thirdly, the age and sex of the subjects was rarely reported. Lastly, and probably most importantly, these studies typically compared autopsy samples of cardiac tissue from the general population who had no evidence of cardiac failure or disease to diabetic patients who were laden with cardiovascular risk factors. It became difficult to establish later if the enhanced atherosclerotic development reported in the diabetic patients was due to the fact that they had diabetes or whether it was due to the accompanying hyperlipidemia, obesity or hypertension. However, more recent studies have firmly established that the diabetic population does suffer from an increased incidence of coronary heart disease which is closely tied to the diabetic disease and myocardial failure (Stout, 1979; Garcia et al, 1974; Hamby et al, 1976; Ostrander et al, 1965; Dortimer et al, 1978; Ledet, 1981).

In contrast to the bulk of the research on atherosclerotic development during diabetes, several post-mortem studies of patients who have died from heart failure have reported an equal incidence of coronary artery disease in the human diabetic and non-diabetic populations (Vihert et al, 1969; Ledet, 1968; Verska and Walker, 1975). The possibility, therefore, of a defect in cardiac performance during diabetes due to a lesion in the muscle itself and not due to coronary vasculature complications was raised and supported by two essential lines of evidence. Firstly, a great number of studies have documented abnormalities in contractile

function of the heart in the absence of major vessel disease in human (Ahmed et al, 1975; Hamby et al, 1974; Regan et al, 1975; 1977; Rubler et al, 1972; 1978; Sanderson et al, 1978; Shah, 1980; D'Elia et al, 1979; Shapiro et al, 1980; Seneviratne, 1977) and animal (Penpargkul et al, 1980; Regan et al, 1974; 1981; Haider et al, 1974; 1981; Fein et al, 1980; 1981; Vadlamudi et al, 1981; Ingebretsen et al, 1980; Miller, 1979; Ku and Sellers, 1982, Senges et al, 1980; Ganguly et al, 1983) diabetic models. Secondly, ultrastructural derangement of the cardiac tissue as observed by electron microscopic analysis, preceded the development of significant vascular lesions (Giacomelli and Weiner, 1979). These data, therefore, have been somewhat convincing in the argument that primary abnormalities may be responsible for cardiac dysfunction in some diabetic patients.

There are three general mechanisms which may be responsible for the primary cardiomyopathy during diabetes. The first, originally proposed by Regan's laboratory, attributes the depressed ventricular performance of diabetic humans and animals to a decreased compliance of the ventricular wall (Regan et al, 1974; 1975; 1977). The enhanced stiffness of the ventricular wall was suggested to account for the depressed end-diastolic volume and stroke volume observed in diabetic humans (Regan et al, 1977) and animals (Regan et al, 1974). This is thought to be due to an increased deposition of PAS-positive staining material, which presumably represents collagen. Collagen content is elevated in myocardium from diabetic animals (Regan et al, 1981).

Many clinical investigations of cardiac function during diabetes have associated contractile dysfunction in the absence of macrovascular complications with microvascular alterations (Shah, 1980 Shapiro et al, 1980; Factor et al, 1980; Blumenthal et al, 1970; Silver et al, 1977;

Rubler et al, 1972; Kannel et al, 1974; Hamby et al, 1974; Seneviratne, 1977; Sanderson et al, 1978). Although this has now emerged as a popular theory to explain cardiac failure during diabetes, little direct analysis of this abnormality has been carried out. The dye-perfusion work of Factor et al (1980) is perhaps most suggestive but even if present, the significance of the microsvascular changes with regard to the pathology of cardiac failure is speculative. In the study of Factor et al (1980), no specific necrosis, fibrosis or other abnormalities were observed in the tissue perfused by the defective vessels. Myocardial degeneration appeared over a widespread area and did not correlate with areas associated with the aneurysm. Furthermore, Shirey et al (1980) found no evidence which would support a role of the microvasculature in cardiac failure in general.

It is, therefore, very plausible that the defect in cardiac performance during diabetes may lie within the cell itself. It is possible that a subcellular malfunction may be responsible for the altered cardiac performance. Such a viewpoint is not unprecedented in the field of cardiac pathophysiology. A subcellular basis for cardiac failure in a wide variety of clinical and experimental settings has been previously proposed (Dhalla et al, 1978; 1982). Since cardiac function is ultimately determined by the membraneous control of cellular ion movements and contractile protein interaction (Dhalla et al, 1977), it is logical to speculate that many of the contractile abnormalities reported previously by other investigators could be as a result of alterations in the functional integrity of these subcellular organelles. It was the purpose of this study, therefore, to examine the function of the sarcolemmal and mitochondrial membrane systems as well as the myofibrillar contractile proteins in the heart during chronic experimental diabetes.

II. REVIEW OF THE LITERATURE

Models of Diabetes

A. Clinical Classification of Diabetes Mellitus

Diabetes mellitus is clinically recognized by the presence of serious abnormalities in carbohydrate metabolism. The manifestation of the disease is characterized by fasting hyperglycemia and/or impaired glucose clearance from the blood after ingestion of a high glucose load. Generally, there exists two major types of diabetes mellitus which, according to the guidelines of the National Diabetes Data Group (1979), are classified as insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM).

IDDM is the classification employed now which encompasses the older terms of Juvenile-Onset, Ketosis Prone, or Type I to describe these patients. It is characterized by an absolute deficiency of insulin which is therefore the cause of the hyperglycemic condition. These patients require insulin to maintain a normoglycemic condition. IDDM most often suddenly presents itself early in childhood but it may also occur in adults (Notkins, 1979). Polydipsia, polyphagia, polyuria and ketosis are frequent symptoms of the disease. Patients are normally thin. The cause of IDDM is unknown at present, therefore it is an incurable disease. However, it is the consensus of scientific and medical opinion to date that the destruction of the beta cells of the pancreas which results in the diabetic symptoms is probably caused by either autoimmune abnormalities or viral infection (Craig, 1980). This may be genetic in origin but more likely IDDM is predominantly a non-genetic disease (Craig, 1980).

NIDDM has been frequently named Maturity-Onset, Non-Ketotic or Type II diabetes in the past. NIDDM patients represent over 80% of

the diabetic population. Its appearance is usually gradual and frequently occurs in adults over 40 years of age, however, it may occur in children too (Craig, 1980). The patients are usually obese, insulin concentrations are frequently quite normal and therefore insulin therapy is not usually required (Craig, 1980). The defect in NIDDM is not pancreatic in origin but more likely related to a cellular resistance to insulin's actions in the body. This may involve receptor and post-receptor lesions in the cell (Kolterman et al, 1980). It is commonly thought to involve some sort of genetic predetermination (Craig, 1980).

B. Animal Models of Diabetes.

Since there are many obvious advantages in employing an animal model with which to study the pathogenesis of any disease, it has become important to develop various disease models in animals which may closely approximate the clinical situation. Diabetes mellitus is no exception to this statement. Generally, the animal models of diabetes can be subdivided into two broad categories: 1) Spontaneous, genetically determined diabetes and 2) Experimentally-induced diabetes. Each type has advantages and disadvantages regarding its use and applicability to the clinical picture, however, unfortunately no model of animal diabetes precisely replicates all of the features of the human diabetic condition (Mordes and Rossini, 1981). It is essential, therefore, that any data or conclusions obtained from animals studies of diabetes be interpreted with a certain amount of caution.

1) Spontaneous hyperglycemia has been discovered and subsequently developed in mice, hamsters, rats, dogs, apes, guinea pigs and swine (Mordes and Rossini, 1981; Lage et al, 1980; Phillips et al, 1982). These animals are either hypo- or hyperinsulinemic and the metabolic

and secondary pathological characteristics vary considerably between all of the animals. Thus, a brief description of each of the most prominent models of diabetes is warranted here.

As is the case with insulin dependent human diabetic patients, hypo-insulinemic diabetic animals are usually lean. Most show beta cell damage and necrosis, and if left untreated will become ketotic and coma-tose. The most commonly employed animal models exhibiting these symptoms are the guinea pig, BB Wistar rat, Chinese hamster, C57BL/KsJ db/db mouse, Celebese black ape, Keeshond dog, Yucatan swine and the NOD mouse (Mordes and Rossini, 1981; Lage et al, 1980; Phillips et al, 1982; Howard, 1982).

The BB Wistar rat was originally discovered in Canada (Nakhooda et al, 1977). Both sexes are affected and its onset is usually around the time of sexual maturation (Marliss et al, 1981). Symptoms of polydipsia, polyuria, glycosuria, ketonurin and tachypnea are all present. Death from ketosis and dehydration can occur in just a few days if left un-attended. These animals are particularly susceptible to pulmonary in-fections, intestinal parasitic attack, and lymphoma proliferation (Marliss et al, 1981). There is evidence of complications suggesting a neuropathy may also be present (Sima, 1980).

The Chinese hamster has about half the life expectancy of its non-diabetic counterparts (Grodsky and Frankel, 1981). Ketosis is present in only 15% of the animals and onset of the diabetic state normally takes about 1-3 months (Grodsky and Frankel, 1981). The animals are frequently hypercholesterolemic and suffer from microangio-pathic but not macrovascular complications (Schlaepfer et al, 1974). Retinopathy, neuropathy, urinary tract dysfunction and infertility are

all associated with the Chinese hamster (Grodsky and Frankel, 1981). An increased incidence and faster onset of spondylosis in the diabetic Chinese hamster has also been reported (Silberberg and Gerritsen, 1976).

The rest of the spontaneously diabetic, hypoinsulinemic animals are not as extensively characterized. The spontaneously diabetic guinea pig exhibits elevated serum triglycerides. Female reproductivity is significantly impaired. Curiously, the cause of the disease may be infectious since even control animals will develop the disease if exposed to the diabetic colony (Lang and Munger, 1976). Both the Keeshond dog and Yucatan mini-swine exhibit plasma hyperlipidemia in addition to the hyperglycemic, hypoinsulinemic condition (Phillips et al, 1982; Kramer et al, 1980). The primate Macaca nigra has been reported to have elevated plasma cholesterol and triglycerides but is rarely ketotic. Atherosclerotic plaque formation has also been observed (Howard, 1982).

Spontaneously diabetic animals which exhibit hyperglycemia and elevated or normal insulin levels, resembling the NIDDM in man, have also been reported. They are frequently obese and are usually found in the rodent family.

The Jackson Laboratory C57BL/KsJ db/db mouse was discovered recently and has been extensively examined. By 4 weeks of age the animals are hyperglycemic, hyperinsulinemic, hyperphagic and obese (Mordes and Rossini, 1981). At 4 months the circulating insulin levels become depressed, the animals lose weight and die quickly. These animals may have thermoregulatory defects (Coleman, 1982), nervous system (Sima and Robertson, 1979; Coleman, 1982) and microvascular disorders (Bohlen and Niggl, 1979). Kidney dysfunction and sterility have also been reported (Gartner, 1978; Coleman, 1982).

The other obese, hyperglycemic, hyperinsulinemic animals models are not as well characterized and unfortunately, most symptoms will reverse themselves during the life span of the animals. The yellow mouse, KK mouse, sand rat, spiny mouse and NZO mouse are typical examples (Mordes and Rossini, 1981).

The advantages and disadvantages of employing the spontaneously diabetic animals limit their usefulness in diabetic research. The obvious advantage of using a spontaneously occurring model of diabetes is that no interventions were necessary to obtain the diabetic state; therefore, it is suggested that these animals may more closely represent the human diabetic model. There are, however, several drawbacks which must be carefully considered. Firstly, since little is known concerning the etiology of the disease in these animals it is only an assumption that this diabetic model is a true representation of human diabetes. Secondly, most of these animals have not been extensively characterized which again lends suspicion to the suggestion of an association with clinical diabetes. More practical concerns also arise with regard to their management and upkeep. Many of these animals require daily insulin injection, sterile environments and constant attention to blood glucose fluctations. The availability of these animals is highly limited and they are usually expensive if available. Depending on the type of experiments to be undertaken, the serious problem of tissue quantity must also be considered because small rodents are the only animals available.

2) Experimentally-induced diabetes in many animal species has been more frequently employed in the diabetic research field. Pancreatectomy is one obvious example but more often diabetes is produced in animals by the injection of an agent which will induce beta cell necrosis of the

pancreas. Consequently, these animals are characteristically hyperglycemic and hypoinsulinemic.

Injection of large quantities of naturally occurring hormones like epinephrine, glucagon, growth hormone and various glucocorticoids have all been shown to produce diabetes in animals (Buse et al, 1957; Mordes and Rossini, 1981). Injection of specific viruses has also been demonstrated to elicit beta cell necrosis and a diabetic condition in mice (Yoon et al, 1982; Craighead and Steinke, 1971; Notkins, 1979). However, two drugs have gained widespread use as diabetogenic agents - alloxan and streptozotocin. Because of their general acceptance in the diabetic field, a comprehensive discussion of their usage was felt to be warranted.

The site of action of alloxan and streptozotocin is the beta cells of the pancreas (Rakienten et al, 1963; Veleminsky et al, 1979). It is important that this toxic action be as specific in nature as possible and therefore certain precautions are undertaken in the preparation and delivery of these agents. Alloxan and streptozotocin are conveniently dissolved in a citrate buffer (pH 4.5) because of their relative instability at an alkaline pH (Patterson and Lazarow, 1949). This also appears to increase the success rate of producing diabetes in rats (Hearse et al, 1975). The half life of streptozotocin in serum is 15 min (Schein et al, 1973) whereas the half life of alloxan is much shorter. In the body it was estimated to be less than 2 minutes (Leech and Bailey, 1945) whereas in vitro measurements found it to be 0.9 minutes (Patterson and Lazarow, 1949). Because of the relatively short half lifes of these drugs, intravenous administration is recommended to avoid undesirable non-specific necrotic effects. They are usually given in a single injection since the animal develops

a certain amount of resistance to repeated injections of lower doses (Like and Rossini, 1976). The rate of injection has also been suggested to influence the diabetogenic effects of alloxan (Pincus et al, 1954).

The response of the body with respect to blood glucose and insulin concentrations is extremely varied after alloxan or streptozotocin injection. Up to 1 hour after injection, the animal suffers from a transient hypoglycemic period (Wrenshall et al, 1950) followed an hour later by a severely hyperglycemic episode (Schein et al, 1971; Junod et al, 1967; Stauffacher et al, 1970). The reason for these fluctuations is unclear since plasma insulin levels are normal (Stauffacher et al, 1970). However, by 6-7 hours post injection a dangerously hypoglycemic period is initiated by a massive release of pancreatic insulin which, if severe enough, will kill the animal (Junod et al, 1967; Stauffacher et al, 1970; Schein et al, 1971). If the animal survives, by 24-48 hours after the injection it will exhibit elevated blood glucose levels which will be chronic in nature.

The cause of this chronically hyperglycemic state after alloxan or streptozotocin injection is beta cell necrosis in the pancreas. As early as one hour after streptozotocin injection, beta cell damage was observed (Junod et al, 1967). By 7 hours post injection when insulin release was high and blood glucose was low, beta cell necrosis was evident (Junod et al, 1967). This observation has prompted the suggestion that the insulin is not actively released during this period but "leaks" from a severely damaged cell (Stauffacher et al, 1970). Twenty-four hours after the initial injection, pancreatic insulin content was less than 1% of control (Veleminsky et al, 1970). Abnormal glucose tolerance was observed when pancreatic insulin was depleted by about 30% but

fasting hyperglycemia and glycosuria were only present when pancreatic insulin was depleted by about 70% (Junod et al, 1969).

The mechanism by which alloxan or streptozotocin elicit their specific necrotic action on the pancreatic beta cells is largely unknown. Like and Rossini (1976) have presented evidence from a study in which they employed multiple subdiabetogenic doses of streptozotocin and examined islet ultrastructure. Their results suggested that the diabetic action of streptozotocin may involve a cell-mediated autoimmune response, which would correlate well with Notkins (1979) theory of a viral-induced human diabetic state. Another hypothesis proposed by Boquist (1980) to explain alloxan diabetogenicity involved a complex interaction with beta cell mitochondrial function. Alloxan was suggested to alter phosphate distribution in the cell, upset intracellular pH and eventually precipitate a condition of excessive Ca^{2+} accumulation in the cellular mitochondria. This would result in an impairment of oxidative phosphorylation function which would ultimately end in cellular necrosis. However, the most current theory involves the ability of alloxan and streptozotocin to generate free radical formation (Malaisse, 1982). It has been hypothesized that the free radicals generated by these agents cause cellular damage and necrosis and the resultant hypoinsulinemic condition (Malaisse, 1982). This contention is supported by data demonstrating that free radical scavengers, like superoxide dismutase, was successful in inhibiting the diabetogenic action of alloxan (Grankvist et al, 1981). This theory may not be incompatible with other theories regarding the mechanism of action of these drugs but it requires further study before conclusive statements can be made.

Several limiting considerations must be kept in mind when employing

the drug-induced diabetic animal model. The susceptibility of animals to streptozotocin is age and sex dependent. Mordes and Rossini (1980) found young rats resistant to all but massive doses of streptozotocin, and as the age of the animal increased, lower streptozotocin doses were needed to elicit a diabetic condition. This may be species dependent, however, since Riley et al (1981) found the exact opposite situation in mice given streptozotocin. Species dependent differences in immunity to alloxan administration (Gorray et al, 1981) and streptozotocin-toxicity dosages (Pitkin and Reynolds, 1970) have been reported. Long term investigations of streptozotocin-induced diabetes in rats must also be undertaken cognizant of a report of spontaneous reversal of the diabetic state in these animals 6-18 months after injection (Tai et al, 1980). Further, the diet of the animal prior to injection can also influence its susceptibility to drug-induced diabetes (Goda et al, 1982; Rerup, 1970).

Certain characteristics of the two drugs alloxan and streptozotocin are very different. Firstly, it is important to recognize that the actions of these agents on the beta cells are quite different (Srivastava et al, 1982). This is emphasized by the observation that only nicotinamide and 3-0-methyl glucose will block the diabetogenic action of streptozotocin (Schein et al, 1967; Ganda et al, 1976) but a number of compounds can block alloxan's action (Ganda et al, 1976). In addition, the toxic effect of alloxan on pancreatic beta cells is much slower than streptozotocin (Veleminsky et al, 1970). The diabetic state is also different. Alloxan diabetes is usually ketotic but streptozotocin diabetes is normally not ketotic

until very high dosages are employed (Mansford and Opie, 1968; Schein et al, 1971). The plasma lipid profile is also very different in the two models of drug-induced diabetes (Veleminsky et al, 1970; Mansford and Opie, 1968). Direct investigation of the non-specific toxic nature of these drugs has resulted in streptozotocin being considered the superior of the two agents (Srivastava et al, 1982; Arison et al, 1967). Alloxan is capable of non-specific lung and kidney damage (Bruckmann and Wertheimer, 1947; Vargas et al, 1970) and injury to the pancreatic alpha cells (Ostenson, 1980). Radiolabelled alloxan was shown to be predominantly taken up by the pancreatic islet cells but its distribution was observed almost throughout the body of mice (Hammarstrom and Ullberg, 1966). Streptozotocin does not appear to elicit these non-specific necrotic effects and, therefore, may be the more appropriate agent to employ in studies of drug-induced diabetes (Agarwal, 1980; Srivastava et al, 1982). Thus, it has been suggested that streptozotocin-induced diabetes bears a closer resemblance to IDDM in humans than does alloxan-induced diabetes (Agarwal, 1980).

The disadvantages of employing drug-induced diabetes therefore, obviously relate to a concern over the nonspecific actions of the drug. However, their usage is convenient, reproducible and the extent of hyperglycemia, ketonemia, etc., easily controlled by varying the dosage of the drug. In addition, most animal species can be studied. The close approximation of streptozotocin-induced diabetes to the human IDDM state would warrant its continued usage in diabetic research.

Cardiac Function During Diabetes

Because it is an obvious and unequivocable indicator of muscle

dysfunction, cardiac contractility measurements are normally carried out to assess the functional integrity of the heart during an experimental condition. Many investigators have studied heart pump function in the diabetic population through the clinical use of a number of methods including phonocardiography, electrocardiography, echocardiography, catheterization techniques for the measurement of vessel pressure, and indicator-dye dilution techniques in order to assess chamber volumes. Each method has advantages, limitations and problems inherent with its use and, therefore, it is advantageous to obtain a picture of cardiac function during diabetes by a number of clinical techniques.

As a pump, the heart of a diabetic patient is in a compromised condition according to most indices of cardiac performance. Stroke volume and cardiac index are depressed in diabetes (Hamby et al, 1974; Regan et al, 1977). The ejection fraction is also lower (Hamby et al, 1974; Seneviratue, 1977; D'Elia et al, 1979). Several studies have reported an increased isovolumic time in diabetic patients (Ahmed et al, 1975; Rubler et al, 1978; Shapiro et al, 1980) which would correspond to an observation of delayed opening of the mitral valve in diabetes (Sanderson et al, 1978). The time of the pre-ejection period was increased during diabetes (Ahmed et al, 1975; Rubler et al, 1978) and the pre-ejection period/left ventricular ejection time ratio was dramatically increased (Regan et al, 1975; Ahmed et al, 1975; Seneviratue, 1977; Rubler et al, 1978, Shapiro et al, 1980) particularly because the left ventricular ejection time was also found to be depressed (Ahmed et al,

1975; Shapiro et al, 1980). Increases in the left ventricular end diastolic pressure and its ratio to chamber volume were observed in diabetic patients (Regan et al, 1977; D'Elia et al, 1979). These indices could indicate a decreased compliance of the left ventricle which would significantly hamper the function of the heart as a pump (Regan et al, 1977). Shapiro et al (1980) also found a slower relaxation process in hearts from diabetic patients.

The use of animals in cardiac contractile studies is particularly advantageous because of the number of parameters which can be more critically and accurately measured. In situ heart preparations, atrial preparations, isolated heart, working heart and papillary muscle experiments were performed employing diabetic animals. In general, the findings with diabetic animal hearts correspond to those obtained in human diabetics which demonstrated a significant depression in contractile performance of the cardiac muscle. Stroke volume, stroke work, cardiac output, peak contractile force, and peak systolic pressure were all found to be depressed in diabetic preparations (Regan et al, 1974; Haider et al, 1977; 1981; Miller, 1979; Penpargkul et al, 1980; Vadlamudi et al, 1982; Ku and Sellers, 1982). The force generation-velocity relationship was also demonstrated to be altered during diabetes. Fein et al (1980) noted an increased time to peak tension and many investigators (Strobeck et al, 1979; Ingebretsen et al, 1980; Vadlamudi et al, 1982; Ganguly et al, 1983; Tahiliani et al, 1983) found depressed +dP/dt or +dT/dt values in cardiac muscle preparations from diabetic animals. Indices of relaxation were also impaired. Relaxation velocity was depressed as demonstrated by -dP/dt or -dT/dt values (Strobeck et al, 1979; Penpargkul et al, 1980; Fein et al, 1980; Vadlamudi et al, 1982; Ganguly et al, 1983; Tahiliani et al, 1983). As was the case in the human work, the observations that

the diabetic animal hearts exhibited increased left ventricular end diastolic pressure (Haider et al, 1981) and decreased end diastolic volume (Regan et al, 1974; Haider et al, 1981) suggests a problem with ventricular compliance may be present.

The results from the heart contractility studies in animal and human diabetic models correlate well. Both sets of results demonstrate an impairment in contractile force generation, a delayed relaxation process and difficulties with respect to ventricular filling in hearts during diabetes.

The response of hearts from diabetic animals to pressure-overload, ischemic or anoxic challenge has also been investigated. Regan et al (1973) elicited an increase in afterload pressure by infusing angiotensin in dogs. While control animals responded with a significant increase in stroke work and end diastolic volume, diabetic dogs did not have any significant change in these parameters. This depressed response of the diabetic animal heart to elevated afterload pressure was confirmed by another study (Ingebretsen et al, 1980). Following anoxic (Hearse et al, 1975; Ingebretsen et al, 1980) or ischemic challenge (Hearse et al, 1978; Feuvray et al, 1979), diabetic rat hearts exhibited a depressed recovery and greater cardiac damage than control hearts. This would provide further evidence to support the compromised functional status of these animals.

The status of coronary flow in experimental diabetes is somewhat unclear. Basal coronary flow rates in diabetic animals have been reported to be significantly increased (Stam and Hulsmann, 1977; Penpargkul et al, 1980), or unaffected (Regan et al, 1973; Vadlamudi et al, 1982). Two other studies report moderate increases in coronary flow in diabetic animals (Haider et al, 1977; Feuvray et al, 1979). Feuvray

et al (1979) also observed elevated coronary flow rates in diabetic rat hearts in response to mild ischemia and transiently lower flow rates in response to a more severe ischemic condition. In the study by Stam and Hulsmann (1977), the investigators demonstrated an increased prostaglandin release in diabetic hearts and a reduction in flow rate after indomethicin infusion. They concluded that the increased coronary flow observed in the diabetic hearts could be accounted for by the enhanced synthesis and release of prostaglandins. This study, coupled with the results of Downing et al (1982) which showed a depressed response by diabetic heart coronaries to adenosine infusion, would suggest coronary flow has become significantly altered during the course of the diabetic disease. This is consistent with a general increase in coronary flow in response to primary myocardial disease in humans (Klassen et al, 1973).

Cardiac Ultrastructure During Diabetes

Although two preliminary reports (Bhan et al, 1978; Factor et al, 1978) have observed no structural derangements at a light microscopic level, several recent studies have demonstrated dramatic changes in myocardial ultrastructure when examined with the electron microscope. Evidence of irregularities of the Z bands, contraction zones and generalized myofibrillolysis have been reported in myocardium from diabetic animals (Tarach, 1976; Giacomelli and Weiner, 1979; Onishi et al, 1981). Increases in mitochondrial area, presumably due to mitochondrial proliferation and swelling, have been observed (Regan et al, 1975; Tarach, 1976; Giacomelli and Weiner, 1979; Onishi et al, 1981). Electron dense mitochondrial deposits and mitochondrial membrane damage have also been shown (Giacomelli and Weiner, 1979; Lebkova et al, 1980; Onishi et al,

1981). Several studies have reported dilatation of the sarcoplasmic reticular tubules as a common feature of the diabetic cardiomyopathy (Regan et al, 1975; Tarach, 1976; Onishi et al, 1981). Focal thickening of the sarcolemma has also been noticed (Giacomelli and Weiner, 1979) as well as a widening of the intercalated disc (Tarach, 1976). Increased lysosomal infiltration has been shown by two separate studies (Giacomelli and Weiner, 1979; Lebkova et al, 1980).

These studies, therefore document extensive subcellular derangement in the myocardium during chronic diabetes. This is probably a gradual process since examination after acute (2 day) periods of diabetes have revealed no cardiac ultrastructural damage (Orth and Morgan, 1962). Thus, these results support the presence of a cardiomyopathic condition during diabetes.

Cardiac Metabolism during Diabetes.

With dramatic alterations in plasma substrate concentrations occurring during diabetes, it is not altogether unexpected to find tissue metabolic derangement during diabetes as well. These abnormalities are evident in all three of the major metabolic pathyways: carbohydrate, lipid and protein.

The primary defect in carbohydrate metabolism in the heart during diabetes is an impairment in the glucose transport across the sarcolemmal membrane into the myocardium (Morgan et al, 1961). In addition to a depression in the rate of transport of glucose into the cell, glucose uptake is limited by a reduced rate of phosphorylation of the glucose within the cell (Morgan et al, 1961). The decrease in phosphorylation appears to occur at two key regulatory stages of glycolysis.

The hexokinase reaction (Das, 1973) and the phosphofructokinase step (Opie, 1968) are inhibited in the heart during diabetes. Inhibition of the hexokinase reaction may occur because of an increase in cellular glucose-6-phosphate concentrations (Randle et al, 1963). Inhibition of phosphofructokinase activity is probably due to increased cellular concentrations of citrate (Opie et al, 1979). This reduction in substrate flow through the glycolytic pathway results in an increase in heart glycogen content (Chen and Ianuzzo, 1982). Glycolysis is further inhibited at a later stage of the process. The oxidation of pyruvate by pyruvate dehydrogenase has been shown to be impaired (Garland et al, 1962) which consequently limits the entry of substrate from the glycolytic pathway into the Kreb's Cycle. This effect, together with a reported alteration in lactate dehydrogenase isoenzyme distribution in the heart (Malathy and Kurup, 1972; Srivastava et al, 1982), may account for the reduction in the lactate : pyruvate ratio in diabetic heart (Opie and Mansford, 1971).

Although lipid metabolism normally represents 60% of the oxygen utilization of the heart, it becomes even more predominant during diabetes (Opie et al, 1979). This is reflected by decreased RQ values of hearts from diabetic animals (Cruikshank and Startup, 1934). Free fatty acid uptake increases in the heart (Opie et al, 1979) which results in enhanced concentrations of acyl CoA (Feuvray et al, 1979), triglycerides (Shipp et al, 1973), and tissue free fatty acids (Garland and Randle, 1964). Not only is triglyceride synthesis in the diabetic rat heart increased but lipolysis is inhibited (Paulson and Crass, 1982) which compounds the problem and results in an accumulation of triglyceride lipid stores in the heart in the form of "droplets". Proliferation of these lipid droplets has been demonstrated in diabetic animal heart

by electron microscopy (Tarach, 1976; Giacomelli and Weiner, 1979). Ketone bodies, if present, are also taken up by the heart at an accelerated rate (Opie et al, 1979).

In order to be oxidized, fatty acids must first move from the cytoplasmic compartment into the mitochondria, a process which requires the presence of carnitine. Since carnitine levels have been shown to be reduced in myocardium from diabetic animals (Feuvray et al, 1979; Vary and Neely, 1982), this may limit oxidative phosphorylation of lipid substrates. Moreover, the increased levels of acyl CoA shown to exist in diabetic myocardium (Feuvray et al, 1979) have the capacity to inhibit the mitochondrial ATP-ADP translocase system (Shug et al, 1975), a process responsible for the transport of ATP out of the mitochondria. Another type of mitochondrial transportation system defect has been reported by a different laboratory examining the malate-aspartate shuttle system in the hearts of diabetic rats (Puckett and Reddy, 1979). They found a reduction in substrate delivery into the mitochondria via the malate-aspartate shuttle. All of these factors together may act to impair aerobic energy production in the heart of the diabetic population. Indeed, Haugaard and Haugaard (1964) found evidence of a depressed synthesis of high energy phosphate compounds from a variety of substrates in heart homogenates from diabetic animals which suggested to them that the mitochondrial oxidative phosphorylation process may be compromised. These data would correlate well with several reports of reduced ATP levels in myocardium from diabetic animals (Allison et al, 1976; Miller, 1979; Opie et al, 1979).

Protein metabolism is also significantly affected in the heart during diabetes. Insulin is known to act as a stimulator of protein synthesis and an inhibitor of protein breakdown (Morgan et al, 1974).

Thus, it has not been surprising to discover diabetes produces an increase in protein degradation (Williams et al, 1980) and a decrease in protein synthesis in the heart (Pain and Garlick, 1974; Williams et al, 1980). This, therefore, would account for descriptions of the process as metabolic "wasting" (Oakley, 1968; Penpargkul et al, 1980) and concur with the high plasma urea levels reported during diabetes (Penpargkul et al, 1980).

Function of Cardiac Subcellular Organelles during Diabetes.

Dillmann (1980) gave the first detailed evidence of contractile protein dysfunction in the heart during diabetes. Ca^{2+}-ATPase activities of myosin and actomyosin fractions were observed to be depressed in hearts from diabetic rats. This was found to be associated with alterations in the myosin isoenzyme composition. The V_1 component, which has the highest mobility and Ca^{2+}-ATPase activity (Hoh et al, 1977), decreased in content in diabetic myosin preparations whereas the V_3 component, which has the slowest mobility and Ca^{2+}-ATPase activity (Hoh et al, 1977), increased in content. These results were essentially confirmed by Malhotra et al (1981). Cardiac myosin heavy chain and actin composition from ventricular tissue of diabetic humans and animals has been reported to be altered (Shoizaki et al, 1979; Maeno et al, 1981). Myosin K^{+}-EDTA ATPase activity was found to be significantly elevated in the diabetic samples (Malhotra et al, 1981). These defects were found to be time-dependent in that they appeared only 1 week after streptozotocin injection. They did not appear to be associated with the caloric deficiency present in the diabetic rats nor were the effects due to a direct cardiotoxic action by streptozotocin (Malhotra et al, 1981). The depressed ATPase activity and the myosin isoenzyme distribution patterns

could be normalized in the diabetic animals upon insulin administration (Dillmann, 1980, Fein et al, 1981). Although the hypothyroid status of the diabetic animals did not appear to be responsible for the defects in ATPase activity (Malhotra et al, 1981; Dillmann, 1982), thyroid hormone administration in pharmacological dosages to the diabetic animals could reverse ATPase activity and normalize myosin isoenzyme distribution patterns (Dillmann, 1982).

Cardiac sarcoplasmic reticular (SR) Ca^{2+} transport has been observed to be depressed in diabetic rats (Penpargkul et al, 1981; Ganguly et al, 1983; Lopaschuk et al, 1983). This has been associated with concomitant defects in Ca^{2+}-stimulated ATPase activity (Ganguly et al, 1983; Lopaschuk et al, 1983), although this observation has not been demonstrated by one study (Penpargkul et al, 1981). SR Mg^{2+}-ATPase activity in diabetic rat heart has been shown to be depressed (Penpargkul et al, 1981) or unaffected (Ganguly et al, 1983). The defect in SR Ca^{2+} transport has been shown to be gradual in onset, appearing 2 weeks after induction of the diabetic condition (Ganguly et al, 1983). It was not normalized by thyroid hormone replacement therapy but it was reversed by chronic insulin administration (Ganguly et al, 1983).

The mechanism responsible for the defect in SR function is probably not related to protein alterations (Ganguly et al, 1983). However, a change in SR lipid composition (Ganguly et al, 1983) and myocardial acylcarnitine content may account for the depressed Ca^{2+} accumulating capacity of cardiac SR from diabetic rats (Lopaschuk et al, 1983).

Mitochondrial function in the heart, has also been found to be disturbed during diabetes. These studies, however, have restricted their examination of mitochondrial respiratory capacity to conditions of

acute diabetes alone. Goranson and Erulkan (1949) first suggested the presence of a defect in mitochondrial oxidative phosphorylative activity in the heart during diabetes. Phosphorylation of creatine in heart homogenates in the presence of succinate or malate was found to be depressed in alloxan-diabetic rats. Insulin could normalize this activity. Similar results were observed by other investigators who also employed heart homogenates from diabetic rats (Haugaard and Haugaard, 1964). They found a decreased synthesis of ATP and ADP in the presence of glucose, fructose and lactate. Recently, Chen and Ianuzzo (1982) have described a decrease in cardiac succinate dehydrogenase activity during diabetes, an effect which was insulin-reversible. These results would suggest a depression in the oxidative capacity of mitochondria from hearts of acutely diabetic animals. When oxidative phosphorylation was directly measured, significant reductions in QO_2 and RCI values were demonstrated (Puckett and Reddy, 1979). These effects were normalized by insulin administration.

The mechanism which may be responsible for these defects is unclear, however, a limitation in substrate availability to the mitochondria has been suggested to contribute to the problem (Kerbey et al, 1976; Puckett and Reddy, 1979). Matrix water space of mitochondria from hearts of diabetic rats was increased (Kerbey et al, 1976) and ultrastructural evidence would support a theory of extensive structural damage (Senges et al, 1980; Lebkova et al, 1980).

No studies to date have directly measured alterations during diabetes in the function or composition of a purified preparation of cardiac sarcolemma. Several studies have presented evidence which suggests significant dysfunction in the cardiac plasma membrane, particularly at the site

of Na^+, K^+-ATPase activity. Onji and Liu (1980) were the first to present evidence to suggest diabetes could be associated with a defect in the Na^+, K^+- ATPase enzyme system. They reported a depression in K^+-pNPPase activity in diabetic dog myocytes, however ouabain binding to the myocyte preparations was not altered. Ouabain-sensitive ^{86}Rb uptake of ventricular slices from diabetic rats has been shown to be depressed (Ku and Sellers , 1982). This effect was insulin-reversible. The sensitivity of the diabetic preparations to the positive inotropic action of ouabain was not altered during diabetes (Ku and Sellers, 1982).

Cardiac Autonomic Neuropathy during Diabetes.

Many studies have documented the presence of a neuropathy during diabetes which has important consequences with respect to cardiac function (for reviews see Ewing et al, 1980; Watkins and Mackay, 1980; Hilsted, 1982). The heart may be partly or wholly denervated, however complete denervation is rare (Watkins and Mackay, 1980). Vagal denervation, which is thought to occur before sympathetic denervation (Watkins and Mackay, 1982) is characterized by postural hypotension, resting tachycardia and heart rate invariation during the Valsalva manoever (Ewing et al, 1980). Autonomic neuropathy has been associated with the occurrence of painless myocardial infarcts in diabetics (Faerman et al, 1977). This is an especially significant clinical problem since a complete absence of pain during a myocardial infarct event (Bradley and Schonfeld, 1962) may result in ambulatory care being summoned later than normal and could partly explain the low survival rate of diabetic patients after a heart attack (Partamian and Bradley, 1965). It has been recognized that autonomic neuropathy in diabetics is associated

with an increase in mortality (Ewing et al, 1976).

The status of the sympathetic nervous system during diabetes is unclear. Plasma catecholamines have been reported to be elevated (Christensen, 1974; Paulson et al, 1980), unaffected (Cryer et al, 1978) or depressed (Christensen, 1972) during diabetes. Cardiac content of norepinephrine has been reported to be decreased (Neubauer and Christensen, 1976) and increased (Paulson et al, 1980; Fushimi et al, 1982) during diabetes. Important alterations at a cellular level have been demonstrated to be present in the adrenergic system during diabetes. Diabetic heart preparations have exhibited a reduced sensitivity to isoproterenol or isoprenaline exposure with respect to contractility measurements (Foy and Lucas, 1976; Heyliger et al, 1982; Ingebretsen et al, 1980) and associated increases in cAMP content and protein kinase activity ratios (Ingebretsen et al, 1981). Alterations in receptor density or adenylate cyclase activity have been suggested to represent two mechanisms which may be responsible for the change in adrenergic responsiveness. A depression in alpha and beta adrenergic receptor number has been reported in hearts from diabetic animals (Savarese and Berkowitz, 1979; Heyliger et al, 1982). This reduction in beta receptor number in the myocardium from diabetic animals has been suggested (Savarese and Berkowitz, 1979) to account for the resting bradycardia observed in diabetic animals (Savarese and Berkowitz, 1979; Cavaliere et al, 1980; Senges et al, 1980). Adenylate cyclase activity, which is responsible for the production of cAMP after adrenergic stimulation, has also been shown to be altered during diabetes. Sodium floride and epinephrine stimulation of adenylate cyclase activity was depressed in preparations from diabetic rats (Menahan et al, 1977).

However cAMP phosphodiesterase activity, which is responsible for the metabolism of cAMP, has also been reported to be depressed during diabetes (Das and Chain, 1972). The net result of the alterations in cAMP production and degradation may be an increase in myocardial cAMP content (Chaudhuri and Shipp, 1973), although this too appears to be a matter of debate (Ingebretsen et al, 1981).

Other investigators have concentrated on the nature of the vagal dysfunction during diabetes. Clinical and experimental work supports the presence of vagal dysfunction during diabetes (Ewing et al, 1980). Stuesse et al (1982) found that vagal stimulation reduced heart rate more in diabetic rats than control. An altered response to cholinergic agonists has also been reported in diabetic preparations. Foy and Lucas (1976) demonstrated a reduced sensitivity to the depressor effect of acetylcholine in diabetic rats. In a thorough investigation, Vadlamudi and McNeill (1983) showed an alteration in the response of diabetic rat hearts to carbachol during the duration of the disease. Early in the disease state there were no differences in the reduction of $^{+}dP/dt$ in the presence of carbachol in the control and diabetic rat hearts. However, 100 days after induction of the diabetic state, animals exhibited a reduced sensitivity to carbachol. By 180 days and up to 1 year, diabetic animals exhibited a supersensitivity to carbachol. Similar results have been reported in another investigation (Tomlinson and Yusof, 1981). It was suggested that defective parasympathetic innervation of the heart could result in a lack of acetylcholine which could lead to the development of postjunctional supersensitivity to exogenously administered cholinergic agents (Vadlamudi and McNeill, 1983). There is some evidence to support such a suggestion. Partial and complete

vagal denervation of atria from diabetic rats has been observed (Tomlinson and Yusof, 1981). Demyelination and axonal degeneration in vagal nerve fibers have been reported in both human and experimental diabetes models (Kristensson et al, 1971; Schmidt et al, 1981). It may be of interest to note that the report of vagal dysfunction in humans was accompanied by serious cardiac dysfunction in two of the three patients studied (Kristensson et al, 1971).

III. METHODS

A. Experimental Design

Male Sprague-Dawley rats weighing approximately 200 g were injected into the femoral vein with either streptozotocin (65 mg/kg body weight) or the buffered vehicle (0.1 M citrate, pH 4.5). These two groups of animals would, therefore, represent the diabetic and control groups, respectively. Animals were maintained on normal rat chow and water ad libitum. Six weeks after streptozotocin injection, random diabetic animals were given daily injections of protamine zinc insulin (approximately 2U/day) subcutaneously. All animals were sacrificed by decapitation 8 weeks after injection of streptozotocin or buffered vehicle. This duration of chronic hyperglycemia is sufficient to produce features of the cardiomyopathic process (Fein et al, 1980; Vadlamudi et al, 1981). Hearts were immediately removed, ventricular tissue freed from any connective tissue, atria and large vessels, and the tissue processed for the isolation of the specific subcellular fraction.

B. Plasma Analysis

Blood was collected in heparinized tubes at the time of sacrifice and plasma was prepared. These plasma samples were stored below $0^{o}C$ and later analysed for glucose (Worthington Statzyme Reagent Kit), cholesterol (Sigma Cholesterol Reagent Kit), insulin and triiodothyronine (T_3) (Amersham Radioimmunoassay Kit techniques) concentrations.

C. Myofibrillar Isolation and Characterization

Myofibrils were prepared according to the procedure of Solaro et al, (1971). Briefly, ventricular tissue was homogenized in a Waring Blender in 4 volumes of a medium containing 0.3 M sucrose, 10 mM imidazole

(pH 7.0) and the resultant slurry centrifuged at 17,300 x g for 20 min. The pellet was suspended in 60 mM KCl, 2 mM $MgCl_2$, 30 mM imidazole (pH 7.0) (Buffer 1) and centrifuged at 750 x g for 15 min. The pellet was washed 4 more times using this procedure until it was finally suspended in Buffer 1 plus 2 mM EGTA and centrifuged again at 750 x g. This pellet was washed twice in a medium containing Buffer 1 plus 0.1% Triton X-100. After recentrifugation at 750 x g, the pellet was washed two more times using Buffer 1 before suspending the final pellet in 0.1 M KCl, 20 mM Tris-HCl (pH 7.0).

Mg^{2+}-dependent (basal) ATPase activity was determined at 30°C in a medium containing (in mM): imidazole, 20 (pH 7.0); $MgCl_2$, 2; Na_2ATP, 2; NaN_3, 10; EGTA, 1.6; KCl, 50, unless otherwise indicated. Myofibrillar protein concentration varied from 400 to 700 μg/ml. Appropriate blank tubes contained all of the above with the exception of the protein. Total ATPase activity was determined in a similar medium as above except that the EGTA was replaced by 1 μM free Ca^{2+}, unless otherwise indicated. Ca^{2+}-stimulated ATPase activity was taken as the difference between the values obtained for total and basal ATPase activities. All reactions were terminated after 5 min by the addition of 1 ml of 12% trichloroacetic acid. These samples were centrifuged and the phosphate in the protein-free supernatant determined by the method of Taussky and Shorr (1953). Free Ca^{2+} was calculated by the method of Katz et al (1970), except that the EGTA was held constant and the total Ca^{2+} concentration was varied. Also, association constants of ATP^{4-} for Mg^{2+} (4.47×10^4) and Ca^{2+} for ATP^{4-} (2.09×10^4) were taken from Vianna (1975). The association constant of 4.47×10^6 was used for Ca^{2+}/EGTA binding (Schwartzenbach et al, 1957).

In certain experiments, KCl and ethylene glycol concentrations were

varied in the incubation medium as outlined previously (Kaldor, 1968; Dowell, 1979). In other experiments, myofibrillar sulfhydryl modification was achieved through pretreatment of the myofibrillar samples with N-ethylmaleimide (NEM) essentially as described (Goodno et al, 1978). Myofibrillar protein (5 mg) was incubated for 25 minutes at 30°C with varying NEM concentrations in a total volume of 0.9 ml. At the prescribed time, the reaction was quenched by the addition of a 0.1 ml of 40 mM dithiothreitol. Control samples were treated in an identical manner except NEM was absent from the incubation medium. An aliquot of the reaction medium was taken within an hour of the modification procedure in order to determine ATPase activities.

Myofibrillar sulfhydryl group content was estimated according to the procedure of Habeeb (1972). An aliquot of myofibrillar protein (1 mg) was dissolved in 3 ml of a solution containing 2% sodium dodecyl sulfate, 80 mM sodium phosphate buffer (pH 8.0) and 0.5 mg/ml EDTA. Initial absorbance at 410 nm was recorded before the addition of 0.1 ml of varying concentrations of 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) which was dissolved in 0.1 M sodium phosphate buffer (pH 8.0). Change in absorbance was recorded over a time span of 15-120 seconds. For the calculation of sulfhydryl content, the final optical density value was subtracted from the initial optical density, and then the net absorbance was employed with a molar absorptivity value of 13,600 M/cm.

D. Sarcolemma Isolation and Characterization.

Sarcolemma-enriched membrane vesicles were prepared by the hypotonic shock lithium-bromide procedure (Dhalla et al, 1981). Ventricular tissue was homogenized in a Waring Blender in 1 mM EDTA, 10 mM Tris-HCl (pH 7.4) and centrifuged at 1000 x g for 10 min. All subsequent centri-

fugations in this procedure were done in an identical manner. The pellet was resuspended in the above buffer, stirred at 0-5°C for 15 min and recentrifuged. The sediment was suspended in 10 mM Tris-HCl (pH 8.0), stirred for 15 min and then recentrifuged. This residue was suspended in 10 mM Tris-HCl (pH 7.4), stirred for 15 min and centrifuged again. The pellet here was suspended in 0.4 M LiBr, 0.4 mM EDTA, 10 mM Tris-HCl (pH 7.4) and stirred for 30 min. After centrifugation, the pellet was suspended in 10 mM Tris-HCl (pH 7.4) and stirred for 15 min. Following centrifugation, the pellet was resuspended in 0.6 M KCl, 10 mM Tris-HCl (pH 7.4) and stirred for 15 min to ensure that all contractile proteins were solubilized. The samples were centrifuged, resuspended in 10 mM Tris-HCl (pH 7.4), stirred for 15 min and given a final centrifugation. The pellet was suspended in 1 mM Tris-HCl (pH 7.4) and assays performed at once.

These membranes obtained by the hypotonic-shock LiBr method have been previously shown to be sarcolemmal in origin (Takeo et al, 1979), are predominantly right-side-out in orientation and contain a substantial basement membrane (Matsukubo et al, 1981). This sarcolemmal preparation has been used by this laboratory and others to study characteristics primarily associated with the cardiac sarcolemmal membrane - beta adrenoreceptors, adenylate cyclase, 5'-nucleotidase, and ouabain-sensitive Na^{+}, K^{+}-ATPase and K^{+}-para nitrophenylphosphatase (K^{+}-pNPPase) activities (Bobik et al, 1980; Kramer et al, 1981; Lamers and Stinis, 1981; Panagia et al, 1981). The hypotonic-shock Li-Br procedure is also a well accepted procedure for the isolation of the plasma membrane from tissues other than cardiac muscle (Cheng et al, 1977; Olson et al, 1981). Biochemical characterization and electron microscopic examinations of the final membrane fraction (Takeo et al, 1979; Matsukubo et al, 1981) have suggested that this preparation has minimal mitochondrial, myofibrillar and microsomal

contamination. To ensure differential contamination was not a factor in the present study, an examination of the activities of routine marker enzymes in the sarcolemmal fraction was carried out. Previously described methods were used to determine K^+-EDTA ATPase activity as a myofibrillar marker (Martin et al, 1982), cytochrome C oxidase as a mitochondrial marker (Wharton and Tzagoloff, 1967) and Ca^{2+}-stimulated ATPase activity as a crude marker of myofibrillar and sarcoplasmic reticular contamination (Bers, 1979). However, to be absolutely sure that any effects which may be observed were not due to any artifacts associated with one particular membrane preparation, another distinctly different method of sarcolemmal isolation was employed.

Because of the reputedly high specific activity of the sarcolemmal marker enzyme, ouabain-sensitive Na^+, K^+-ATPase, the isolation procedure of Philipson and Nishimoto (1982) was employed. From 5-10 hearts were homogenized in a Waring blender in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.6), 0.3 M KCl, 25 mM NaP_2O_7 and then washed and centrifuged at 177,000 x g for 20 min twice. The pellet was resuspended in the 250 mM sucrose, 1 mM DTT, 10 mM Tris-maleate (pH 7.6). DNase I (30,000 Kunitz units) was added to the mixture and stirred slowly at room temperature for 1 hour. This vesicular preparation was then disrupted by Polytron homogenization and centrifuged at 13,000 x g for 15 min. The resultant supernatant was saved and centrifuged further at 177,000 x g for 45 min. The sediment from this spin was suspended in 45% sucrose, and bottom layered in a discontinuous gradient consisting of 11, 26, 29, 32, 34% sucrose. The tubes were centrifuged for 16 hrs at 122,000 x g and the vesicles collected as a disperse band in the 26% sucrose fraction. The final sarcolemmal vesicles pelletted at 177,000 x g and were resuspended 0.14 M KCl, 5 mM Tris-maleate (pH 7.4).

Sarcolemmal Mg^{2+}-dependent ATPase activity was determined at 37°C in 1 ml of a medium containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$, 4 mM Tris-ATP and 50 μg protein. Total ATPase activity was that activity observed in a medium as above with the addition of 100 mM NaCl and 10 mM KCl. Na^+, K^+-ATPase activity was taken to be the difference between total and basal activities. Ouabain-sensitive Na^+, K^+-ATPase activity was calculated to be the ATPase activity which was inhibited by 2 mM ouabain. Ca^{2+}-dependent ATPase activity was determined at 37°C in a medium containing 1.25 mM $CaCl_2$, 50 mM Tris-HCl (pH 7.4), 4 mM Tris-ATP and 50 μg protein. Reactions were terminated after 10 min by the addition of 1 ml 12% trichloroacetic acid. These assay conditions have been found to be optimal and are similar to those employed earlier (Takeo et al, 1979). The release of inorganic phosphate was measured in the protein-free supernatant as described (Taussky and Shorr, 1953).

Basal p-nitrophenylphosphatase (pNPPase) activity was measured in a medium containing 100 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 1 mM Na_2-EDTA and 3 mM p-nitrophenylphosphate (fresh) (Lamers et al, 1978). Total pNPPase activity was measured in a similar medium with the addition of 15 mM KCl. K^+-stimulated pNPPase activity was considered to be the difference between the total and basal activities. Ouabain-sensitive K^+-pNPPase activity was calculated as the K^+-pNPPase activity inhibited in the presence of 2 mM ouabain. Paranitrophenol concentrations were measured spectrophotometrically at 405 nm (Lamers and Stinis, 1981).

5'-nucleotidase activity was determined from the rate of conversion of ^{14}C-AMP into ^{14}C-adenosine as outlined earlier (Avruch and Wallach, 1971). Sarcolemmal vesicles (25 μg) were added to 250 μl (final volume) of a medium containing 50 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 0.2 mM ^{14}C-AMP and 2 mM dicyclohexylammonium para-nitrophenylphosphate.

The reaction was terminated after 10 min incubation at $37^{o}C$ by the addition of 50 μl each of 0.25 M $ZnSO_4$ and 0.25 M Ba $(OH)_2$. Samples were centrifuged and an aliquot of the supernatant aspirated and radioactivity measured.

In some cases membranes were pretreated with various agents prior to monitoring the ATPase activities. Alamethicin (ALA) treatment of sarcolemmal membranes was carried out by incubating 1 mg membrane for 20 min at $20^{o}C$ with 1 mg ALA as described (Jones et al, 1980). Samples were placed on ice to terminate the reaction. ALA was dissolved in ethanol and appropriate controls containing similar ethanol concentrations were included. Ethanol in the concentration used in this study had no effect on enzyme activities. Deoxycholate (DOC) treatment of sarcolemmal membranes was done by suspending vesicles in 50 mM Tris-HCl (pH 7.4), 20 mM KCl at a final concentration of 0.2 mg DOC/mg sarcolemmal protein. After mixing, samples were incubated for 10 min at $30^{o}C$ and the reaction terminated by the addition of 2 ml ice-cold 1 mM Tris-HCl (pH 7.4). These samples were then washed in 1 mM Tris-HCl (pH 7.4). Control tubes contained all components except DOC. The concentrations of these agents have been found to produce maximal increases in Na^+, K^+-ATPase activity (Jones et al, 1980; Panagia et al, 1982). Filipin treatment of heart membranes was carried out as outlined (Lad et al, 1979). Filipin was dissolved in methanol and aliquots containing 10, 50, 100 and 200 μg of filipin were placed in separate tubes. Control tubes contained an appropriate aliquot of methanol alone. The methanol was then evaporated by a fine stream of N_2 to leave a thin film of filipin along the sides of the tubes. One mg of sarcolemmal protein was added to each tube, mixed and allowed to incubate at $25^{o}C$ for 10 min. The reaction was stopped by placing the tubes on ice. The membranes were

washed and suspended in 1 mM Tris-HCl (pH 7.4).

ATP-independent calcium binding by the sarcolemmal membranes was studied by the Millipore filtration technique. Membranes (150-200 μg) were incubated at 37°C for 5 min in 1 ml of a medium containing 50 mM Tris-HCl (pH 7.4), 0.05 mM or 1.25 mM $^{45}CaCl_2$. In certain experiments, membranes were pre-treated with neuraminidase (specific activity 5U/mg protein) prior to the determination of calcium binding. Specifically, 2 mg sarcolemmal protein were incubated for 20 min at 37°C in a medium containing 50 mM Tris-HCl (pH 7.4), 20 mM KCl and 20 or 200 μg neuraminidase which represents a ratio of 0.05U or 0.5U neuraminidase/mg sarcolemmal protein, respectively. These concentrations are similar to those employed previously in studies on cardiac sarcolemmal membranes (Takeo et al, 1980; Matsukubo et al, 1981). An aliquot of this medium was then added to the incubation medium mentioned above for the determination of Ca^{2+} binding. After termination of the Ca^{2+} binding reaction by filtration, an aliquot (100 μl) of the filtrate was added to appropriately prepared scintillation vials and the ^{45}Ca radioactivity estimated in a Beckman scintillator spectrometer.

Sialyltransferase activity was determined in the sarcolemmal fraction by the disk method of Baxter and Durham (1979) by incubating 0.3 mg membrane protein for 90 min at 37°C in 100 μl of a medium containing 50 mM imidazole-HCl (pH 7.0), 5 mM $MgCl_2$, 0.5 mg of desialylated human α_1-acid glycoprotein and 1.85 x 10^{-6} mM CMP-sialic acid (170 mCi/mmol). This medium also contained 0.05 or 0.1 mM Na_2-ATP to avoid the influence of any endogenous phosphatase activity. The reaction was terminated by placing the tubes on ice. Sixty μl aliquots were spotted on 2.5 cm Whatman No. 1 filter disks and immersed in 10% TCA. The disks were washed three times in 10% TCA, twice in ethanol: ether (2:1) (v/v) and finally in ether.

The disks were dried, immersed in 1 ml of 0.05 M H_2SO_4 and incubated at 80^oC for 1 hr. The H_2SO_4 was neutralized with 1N NaOH before counting in scintillation medium.

E. Isolation and characterization of mitochondria.

Mitochondria were isolated from ventricular tissue by the method of Sordahl et al. (1971) as previously described in detail (Lee and Dhalla, 1976). Ventricles were suspended in a medium containing 0.25 M sucrose, 1 mM EDTA, 20 mM Tris-HCl (pH 7.0). The tissue was homogenized in a Waring Blender for 20 sec in 10 volumes of KEA medium (0.18 KCl, 10 mM EDTA, 0.5% bovine serum albumin, pH 7.4). The homogenate was centrifuged at 1000 x g for 10 min to remove heavier subcellular organelles and the resultant supernatant further centrifuged at 10,000 x g for 20 min. The pellet was suspended in KEA medium and centrifuged two more times at 10,000 x g. The final 10,000 x g pellet was suspended in 0.25 M sucrose, 25 mM Tris-HCl (pH 7.0) and assays were run immediately after the isolation procedure was completed. In the case of the oxidative phosphorylation studies, samples were suspended in the standard KEA medium. In order to ensure the relative purity of these membranes, biochemical characterization of the fraction was carried out employing marker enzyme techniques which have been previously described in this manuscript.

Evaluation of the oxidative phosphorylative capacity of the mitochondria was accomplished through the use of a Clark oxygen electrode coupled to a Gilson Oxygraph apparatus. The incubation medium of 2 ml was maintained at 25^oC and consisted of 9 mM K_2HPO_4, 225 mM sucrose, 9 mM Tris-HCl (pH 7.4) and 1-3 mg mitochondrial protein. Pyruvate/malate (5 mM) or glutamate (5 mM) was used as substrate and active respiration initiated by the addition of 444 nmoles ADP. The oxygen solubility was assumed to

be 240 nmol/ml at 25°C (Sordahl et al, 1971). All parameters of mitochondrial respiration were measured as outlined elsewhere (Van Jaarsveld and Lochner, 1982).

Mg^{2+}-dependent ATPase activity was determined at 37°C in 1 ml of a medium containing: 100 mM KCl, 10 mM $MgCl_2$, 4 mM Tris-ATP, 20 mM Tris-HCl (pH 6.8) and 150 μg membrane protein. The reaction was started after a 3 min pre-incubation period by the addition of ATP and terminated after 5 minutes by 1 ml ice-cold TCA. Samples were centrifuged and inorganic phosphate determined in the protein-free supernatant as described elsewhere (Taussky and Shorr, 1953).

Ca^{2+} binding and uptake were measured according to the Millipore filtration technique (Lee and Dhalla, 1976). In order to measure Ca^{2+} binding, mitochondrial membranes (200 μg protein) were incubated at 25°C in a similar medium as that described for Mg^{2+}-ATPase activity except for the addition of 100 μM $^{45}CaCl_2$. Mitochondrial Ca^{2+} uptake activity was monitored at 37°C in a similar medium with the addition of 4 mM K_2HPO_4 and 5 mM succinate. Unless otherwise indicated, reactions were terminated after 5 min of incubation by filtration through the Millipore filtration apparatus. The $^{45}CaCl_2$ remaining in the filtrate after filtration was measured in a scintillation counting spectrometer.

F. Protein Analysis.

Protein was quantitated in the various subcellular fractions by the method of Lowry et al (1951). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretic separation of protein was accomplished according to two different protocols. SDS gel electrophoresis was performed on isolated myofibrillar and sarcolemmal preparations as per the method of Weber and Osborne (1969). The gel composition was 0.78% NaH_2PO_4, 3.8% Na_2HPO_4,

0.2% SDS, 0.3% ammonium persulfate (w/v), 0.001% TEMED (v/v) and 10% acrylamide with the acrylamide: N,N'-methylenebis acrylamide at a ratio of 34.8 : 1. Gels were cast in tubes and samples were run at 50 mamps/6 tubes using a Buchler Polyanalyst with .26% NaH_2PO_4, 1.26% Na_2HPO_4, 0.07% SDS employed as an electrode buffer. Fixation was achieved by placing the gels in a 40% methanol, 7% glacial acetic acid (v/v) solution overnight. Staining was accomplished in a solution containing 0.25% (v/v) Coomassie brilliant blue. Destaining was carried out in a solution containing 5% methanol, 7.5% glacial acetic acid (v/v). The resulting bands were scanned at 550 nm in a Unican SP 1800 Spectrophotometer. Molecular weights of the protein peaks were estimated by comparing their mobility with known molecular weight standards run under identical conditions. Protein peaks were quantified according to the method of Potter (1974).

Slab-gel electrophoresis was done on mitochondrial protein samples in an 8.5% acrylamide gel according to the methodology of Laemmli (1970). The separating gel consisted of 0.1% SDS (w/v), 375 mM Tris-HCl (pH 8.8), 0.025% ammonium persulfate (w/v) and 0.005% TEMED (v/v) with the acrylamide: N,N'-methylenebis acrylamide at a ratio of 36.5 : 1. Membrane and molecular weight standard proteins were first applied to a short 4% stacking gel cast on top of the separating gel. The electrode buffer consisted of 0.25 M Tris, 0.2 M glycine, 0.1% SDS (w/v) (pH 8.3). Gels were fixed and stained in a solution of 0.1% Coomassie brilliant blue (w/v), 40% methanol, 15% glacial acetic acid (v/v). Destaining of the gels was completed in a solution consisting of 12.5% isopropanol, 10% acetic acid (v/v).

G. Membrane Lipid Analysis

Membrane protein (2.0 mg) was suspended and lipids extracted overnight in a 2:1 solution of chloroform/methanol (Folch et al, 1957). For the determination of membrane cholesterol, 200 μl samples were taken from the organic lipid extract and analyzed for total cholesterol using the Sigma Cholesterol Reagent Kit. The non lipid contaminants were removed by repeated washings in 75:25:2 chloroform: methanol: HCl. Following drying over nitrogen, the extracted lipids were spotted on activated thin layer chromatography plates and the plates were run in a chromatography tank containing chloroform: methanol: 7N ammonium hydroxide (12:7:1) for 2 hrs. Following drying, the plates were placed in a second tank containing chloroform: methanol: glacial acetic acid: H_2O (80:40:7.4: 1.2) at ninety degrees respective to their position in the first solvent and run for a further 2 hrs (Pumphrey, 1969). Spots were visualized by spraying with H_2SO_4 (5%) and heated for 15 min at 180°C. The phospholipid spots were visualized under ultraviolet light, scraped into labelled tubes and hydrolyzed in 0.7 ml perchloric acid for 2 hrs at 160°C. Inorganic phosphate content was estimated according to the procedure of Bartlett (1969).

H. Sarcolemmal Sialic Acid Determination.

Sarcolemmal membranes (1 mg) were washed and hydrolysed in 0.5 ml of 0.2 N H_2SO_4 at 80°C in order to liberate sialic acid. Sialic acid was separated and its content colorimetrically determined by the thiobarbituric acid assay of Warren (1963).

I. Materials.

Alamethicin was a generous gift from Dr. J.E. Grady, Upjohn Co.,

Kalamazoo, U.S.A. Filipin was also kindly donated by W.A. King, Upjohn Co., Don Mills, Ontario. All other reagents and chemicals were of standard analytical grade. Radioactively labelled compounds were purchased from New England Nuclear, Boston, U.S.A., or Amersham.

J. Statistical Treatment.

Statistical analysis was accomplished by student's t-test or, where appropriate, multiple analysis of variance. Post hoc evaluation of significance was achieved through the use of Duncan's New Multiple Range Test. A P value less than 0.05 was considered to reflect a significant difference.

IV. RESULTS

A. General Features of Steptozotocin-induced Diabetes in Rats.

Experimental animals exhibited diabetic symptoms when examined eight weeks after streptozotocin injection (Table 1). Body and ventricular growth was significantly retarded in the streptozotocin-injected animals. The ventricular : body weight ratio suggested that the hearts from these animals were in a state of hypertrophy. Plasma glucose concentrations were markedly elevated and plasma insulin depressed in comparison to control animals. This data firmly establishes the presence of a severely diabetic state in the experimental animals. Furthermore, measurement of plasma triiodothyronine (T_3) and cholesterol levels in diabetic rats revealed the presence of accompanying hypothyroid and hypercholesterolemic conditions. Control values reported in this investigation for all of the above parameters are similar to those previously described in the literature (Fein et al, 1980; Penpargkul et al, 1980; Vadlamudi et al, 1981; Turlapaty et al, 1980; Ganguly et al, 1983). In addition, these general features of chronic streptozotocin-induced diabetes are in accord with data observed in other laboratories employing a similar experimental protocol (Fein et al, 1980, Penpargkul et al, 1980; Malhotra et al, 1981; Turlapaty et al, 1980). Daily injection of the diabetic rats for 2 weeks with insulin prior to sacrifice result in normalized plasma glucose, insulin, T_3 and cholesterol concentrations. However, this protocol was not of a long enough duration to reverse all of the effects of the diabetic state as evidence by the presence of morphometric alterations still in existence in these rats in comparison to control.

As shown in Table 2, the diabetic condition did not affect the protein yield of two of the three fractions isolated. Both cardiac myofibrillar and sarcolemmal protein yield did not differ among the control,

Table 1. Body, ventricular weight and plasma characteristics from control, diabetic and insulin-treated diabetic rats.

	Control	Diabetic	Diabetic + Insulin
Body Weight (g)	443 ± 5.7 (70)	276 ± 6.7 (82)*	343 ± 7.8 (29)*
Ventricular Weight (g)	1.09 ± 0.01 (53)	0.78 ± 0.02 (67)*	0.89 ± 0.03 (16)*
Ventricular/Body Wt. Ratio (mg/g)	2.48 ± 0.05 (41)	2.88 ± 0.03 (48)	2.66 ± 0.03 (12)*
Plasma glucose (mg%)	131.4 ± 4.5 (16)	475.8 ± 14.6 (17)*	144.2 ± 11.8 (16)
Plasma insulin (μU/ml)	25.5 ± 4.9 (10)	12.8 ± 0.8 (16)*	36.2 ± 5.9 (9)
Plasma T_3 (mg%)	82.0 ± 12.5 (4)	36.0 ± 5.0 (6)*	74.5 ± 8.9 (6)
Plasma cholesterol (mg%)	56.8 ± 4.0 (11)	97.1 ± 7.2 (13)*	62.8 ± 9.9 (6)

Values represent mean ± S.E. Number in parentheses is indicative of sample replication. * $P < 0.05$ vs control.

Table 2. Protein yield of various subcellular organelles isolated from hearts of control, diabetic and insulin-treated diabetic rats.

Fraction	Control	Diabetic	Diabetic + Insulin
Myofibril	43.22 ± 1.1 (18)	39.06 ± 1.1 (18)	46.10 ± 5.2 (7)
Sarcolemma	3.30 ± 0.24 (19)	3.07 ± 0.18 (19)	2.96 ± 0.28 (9)
Mitochondria	5.88 ± 0.25 (12)	7.13 ± 0.38* (12)	5.79 ± 0.17 (8)

Values represent the mean ± S.E. and are expressed in mg protein per gram wet weight of ventricular tissue. Numbers in parentheses represent the number of experiments. * $P < 0.05$ vs control.

diabetic or insulin-treated diabetic groups. However, cardiac mitochondrial protein yield was significantly increased from diabetic rat heart samples in comparison to control. In vivo administration of insulin to the diabetic rats reversed this effect. The control values observed in the present study for protein yield of the various subcellular organelles are similar to those found elsewhere (Dowell, 1979; Panagia et al, 1981; Penpargkul et al, 1978).

B. Cardiac Myofibrillar Function in Diabetic Rats.

In order to determine if the myofibrillar fractions which were isolated from the three groups may be subject to differential contamination, a biochemical characterization of myofibrillar purity was undertaken (Table 3). Mg^{2+}-dependent and Ca^{2+}-stimulated ATPase activities were significantly depressed in cardiac myofibrils from diabetic rats in comparison to the control animals. This effect was normalized upon treatment of the diabetic rats with insulin. The control values for myofibrillar Mg^{2+}-ATPase and Ca^{2+}-stimulated ATPase activities are similar to those reported elsewhere in the literature (Dowell, 1979; Holroyde et al, 1979). These enzymatic activities were found to be completely insensitive to 5 mM NaN_3 which suggests that this myofibrillar preparation contained little mitochondrial contamination. Furthermore, this fraction displayed no detectable oxalate-supported Ca^{2+} uptake or ouabain-sensitive Na^+, K^+-ATPase activity which is strong evidence that neither sarcoplasmic reticulum nor sarcolemma were present in this contractile protein preparation. Since none of the groups exhibited any detectable azide-sensitive ATPase activity, Ca^{2+} uptake or Na^+, K^+-ATPase activity, it appears reasonable to conclude that myofibrils isolated from all of the groups were of a similar degree of purity.

Table 3. Biochemical characterization of myofibrillar fraction isolated from hearts of control, diabetic and insulin-treated diabetic rats.

Activity	Control	Diabetic	Diabetic + Insulin
Mg^{2+}-ATPase	0.221 ± .0099	0.169 ± .013*	.192 ± .011
Ca^{2+}-stimulated ATPase	0.901 ± .032	0.673 ± .050*	.870 ± .020
NaN_3-sensitive Mg^{2+}-ATPase	ND	ND	ND
NaN_3-sensitive Ca^{2+}, Mg^{2+}-ATPase	ND	ND	ND
Oxalate-supported Ca^{2+} uptake	ND	ND	ND
Ouabain-sensitive Na^+, K^+-ATPase	ND	ND	ND

Values represent mean ± S.E. of 4-8 experiments. Activity is expressed in μmol Pi/mg/5 min. * $P < 0.05$ vs control. ND- not detectable. Lower limits of detection for Ca^{2+} uptake and ATPase activity are in the picomolar range.

Contractile protein interaction ultimately determines contractile force generation in any muscle (Mannherz and Goody, 1976). It is possible, therefore, that any change in myofibrillar protein composition may alter aspects of this interaction and consequently will be responsible for a cardiodepressive condition. Since a preliminary study has demonstrated changes in contractile protein composition of diabetic human heart (Shiozaki et al, 1979), we examined cardiac myofibrillar protein composition in chronic streptozotocin-induced diabetes. As shown in Figure 1, SDS-gel electrophoresis of the myofibrillar fractions from control and diabetic rat heart revealed no significant differences in composition. This is in accord with results given by Malhotra et al (1981) using myosin and actin isolated from experimentally-induced diabetic rats.

Mg^{2+}-dependent ATPase activity of myofibrils isolated from control, diabetic and insulin-treated diabetic rat heart was examined across varying incubation times (Figure 2). Mg^{2+}-ATPase was depressed at all time points examined in diabetic preparations and in vivo insulin treatment of the diabetic rats reversed this effect.

A time course study of myofibrillar Ca^{2+}-stimulated ATPase was also undertaken (Figure 3). Myofibrils isolated from diabetic rat heart exhibited significantly lower Ca^{2+}-stimulated ATPase activity than control preparations at all time points examined. Insulin administration to the diabetic rats resulted in a normalization of these activities. The insulin reversible nature of the defects in myofibrillar ATPase activities correlates well with studies which have demonstrated that insulin treatment can normalize contractile function and actomyosin and myosin ATPase activities (Fein et al, 1981).

In order to examine the Ca^{2+} dependency of the myofibrillar Ca^{2+}-

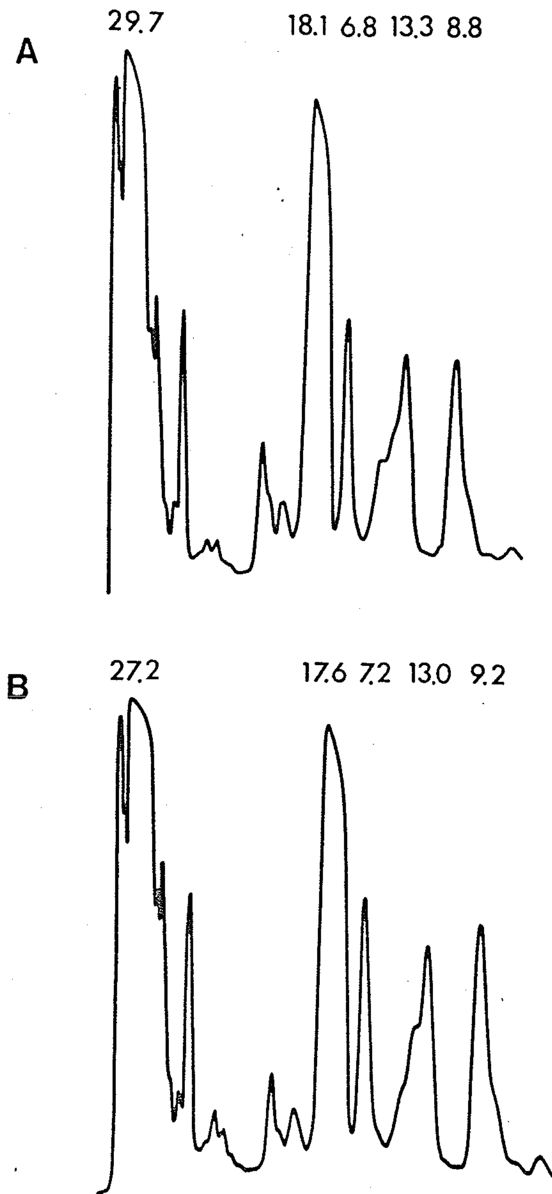

Figure 1. Densitometric scans of myofibrillar proteins separated by SDS polyacrylamide gel electrophoresis. Separation was run in 10% acrylamide gel in the presence of appropriate molecular weight standards (Sigma). A. Control myofibrils. B. Diabetic myofibrils. Values above each protein peak are representative of percentage contribution of that peak to the total myofibrillar protein present.

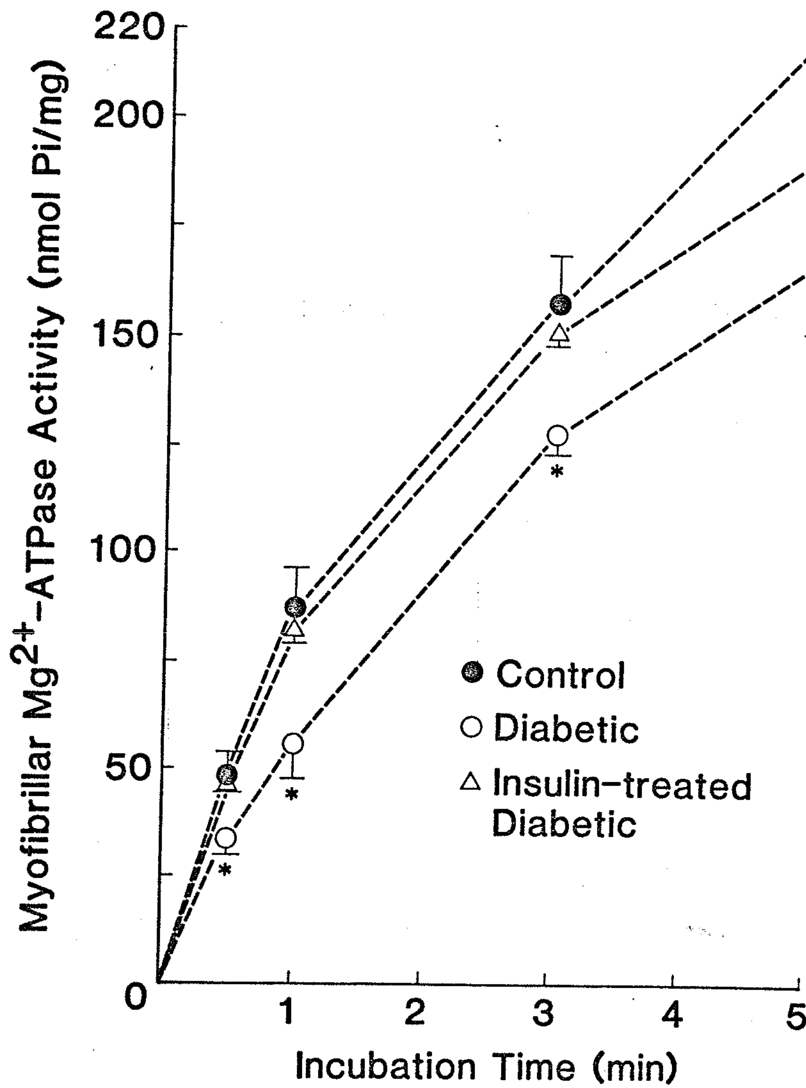

Figure 2. Myofibrillar Mg^{2+}-ATPase activity examined over various times of incubation in control, diabetic and insulin-treated diabetic preparations. Values represent mean ± S.E. of 5 experiments. * $P < 0.05$ vs control as determined by ANOVA techniques.

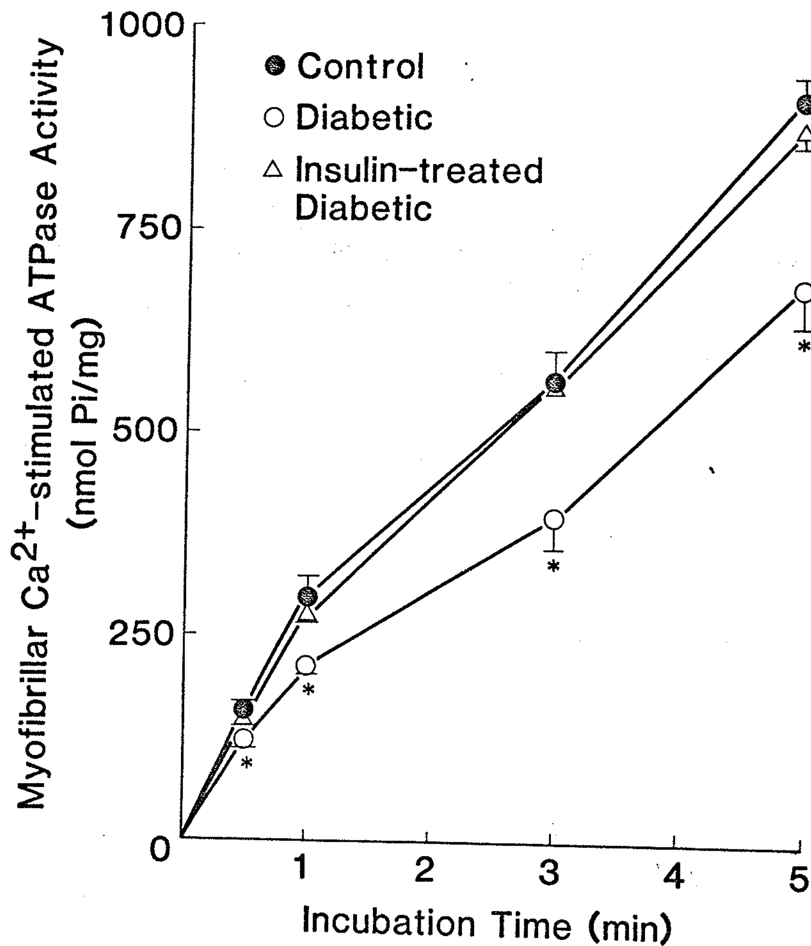

Figure 3. Myofibrillar Ca^{2+}-stimulated ATPase activity examined over various times of incubation in control, diabetic and insulin-treated diabetic preparations. Values represent mean ± S.E. of 5 experiments. * $P < 0.05$ vs. control values as determined by ANOVA techniques.

stimulated ATPase enzyme during diabetes, the free Ca^{2+} concentration in the incubation mixture was varied (Figure 4a). A significant depression in Ca^{2+}-stimulated ATPase activity was observed at all free Ca^{2+} concentrations studied except at 0.10 μM Ca^{2+}. When the enzyme activity of each group was expressed as a percentage of the respective maximal activity seen at 10 μM concentration of Ca^{2+}, the dependence of the normalized myofibrillar ATPase on free Ca^{2+} concentration was the same in control and experimental preparations (Figure 4b). The concentration of free calcium required for half maximal activity in both preparations was about 5×10^{-7}M.

In an effort to further characterize the depression in basal and Ca^{2+}-stimulated ATPase activities in myocardial tissue from diabetic rats, ATPase activities were determined in the presence of varying concentrations of KCl. Results obtained demonstrate the basal ATPase activity in control myofibrillar preparations were depressed by about 40% at 100 mM KCl and a three-fold increase in KCl concentrations failed to elicit further decreases in the activity (Figure 5a). In contrast, basal ATPase activity in myocardial myofibrils isolated from diabetic rats demonstrated a progressive decline in the activity with increasing KCl concentrations. A KCl concentration of 300 mM almost completely inhibited basal ATPase activity in diabetic preparations, a response which was significantly different than that of control preparations. Myofibrillar Ca^{2+}-stimulated ATPase activity in control animals was progressively depressed by increasing KCl concentrations (Figure 5b). Qualitatively, the response of diabetic Ca^{2+}-stimulated ATPase activity to KCl was similar to that of the control; however, quantitatively the Ca^{2+}-ATPase activity in diabetic rats was significantly lower than control values in the presence of 100 and 200 mM KCl levels.

Since ethylene glycol has been demonstrated to detect conformational changes at or near the active site of ATP hydrolyzing enzymes (Kaldor,

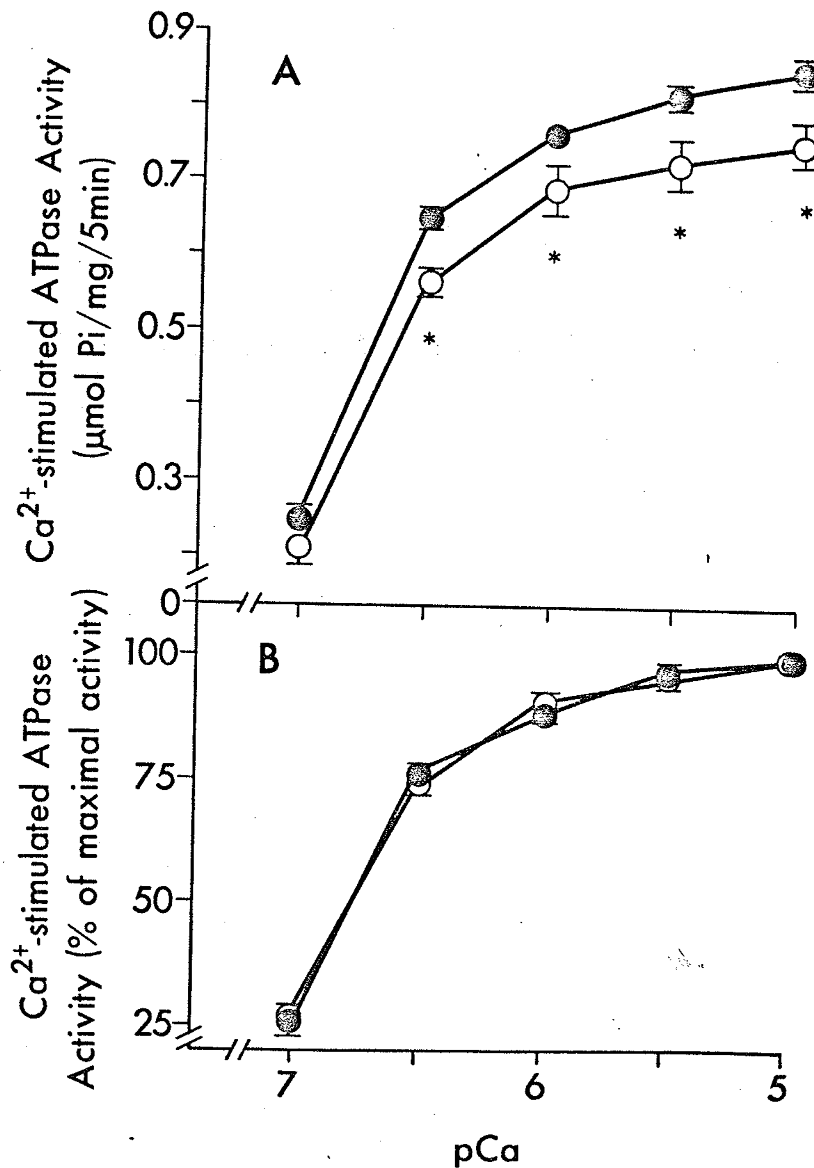

Figure 4. Cardiac myofibrillar Ca^{2+}-stimulated ATPase activity in control (●) and diabetic (O) animals. (a) Absolute Ca^{2+}-stimulated ATPase activity values at varying free Ca^{2+} concentrations. (b) Ca^{2+} sensitivity of Ca^{2+}-stimulated ATPase activity expressed as percentage of respective maximal values at 10 μM Ca^{2+}. *$P < 0.05$ vs. control values ($n = 5$)

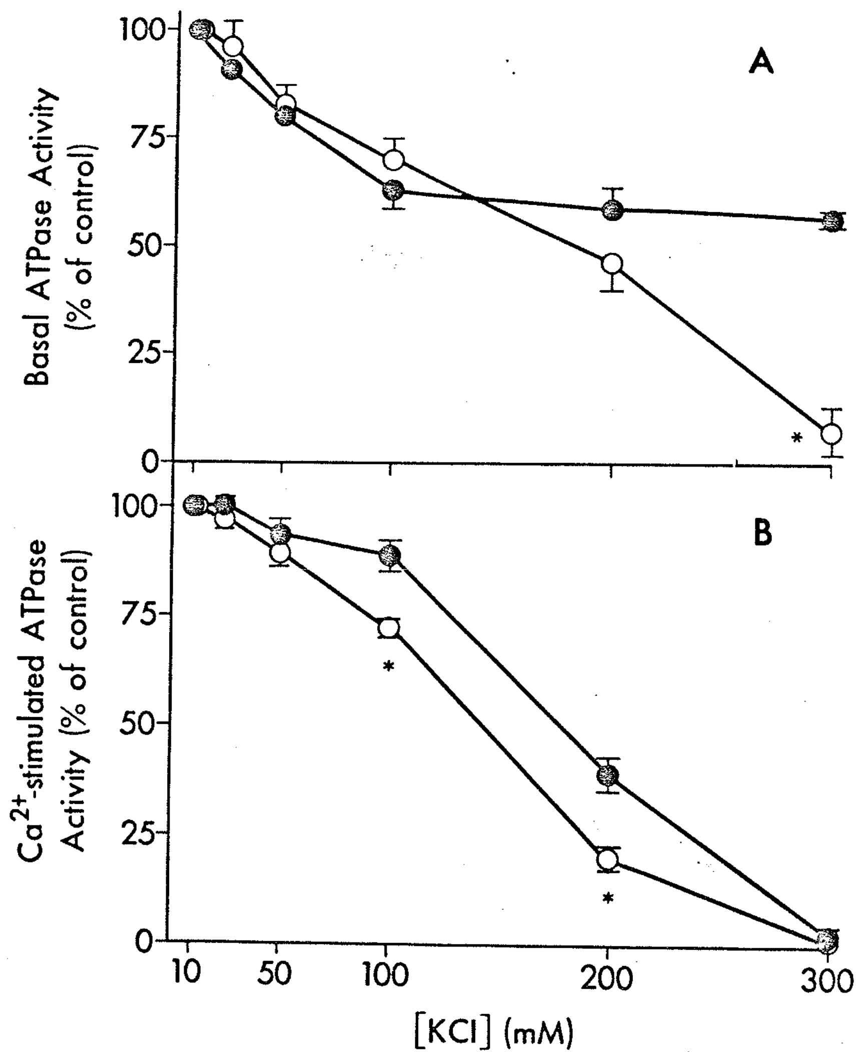

Figure 5. Cardiac myofibrillar ATPase activity from control (●) and diabetic (O) animals in the presence of varying KCl concentrations. (a) Basal ATPase activity; and (b) Ca^{2+}-stimulated ATPase activity. The values are expressed as percentage of respective control at 10 mM KCl. Basal ATPase values in the presence of 10 mM KCl were 0.26 ± 0.02 and 0.18 ± 0.01 μmol Pi/mg/5 min for control and diabetic myofibrillar preparations. *$P < 0.05$ vs. control values (n = 5).

1968), further biochemical characterization of the defect in diabetic myofibrillar ATPase activity was accomplished in which ethylene glycol concentrations in the incubation medium varied from 5 to 40% (v/v). As shown in Figure 6, control basal ATPase activity was elevated in the presence of ethylene glycol; maximal activation occurring at 20% ethylene glycol levels. In contrast, the sensitivity of diabetic myofibrillar ATPase activity to ethylene glycol was greater and ATPase activity continued to increase at concentrations greater than 20%. Myofibrillar Ca^{2+}-stimulated ATPase activities in control and diabetic preparations were depressed by increasing ethylene glycol concentrations and this response to ethylene glycol did not differ significantly between the two groups.

In order to confirm the presence of conformational alterations in myofibrils from diabetic rats and obtain more specific information on the nature of this change, myofibrillar ATPase activities were examined in the presence of N-ethylmaleimide (NEM), a specific sulfhydryl group modifier (Sekine et al, 1962). Increasing concentrations of NEM from 10 to 100 μM enhanced Mg^{2+}-ATPase activity in a dose dependent fashion in control and diabetic preparations (Figure 7). However, at the highest NEM concentration employed, Mg^{2+}-ATPase activity was signficantly lower in diabetic myofibrils than in controls. Ca^{2+}-stimulated ATPase activity was consistently depressed in the presence of increasing concentrations of NEM and the diabetic and control responses to this agent were not statistically different.

Since thiol modification may be implicated from the above results in the defect in Mg^{2+}-ATPase activity in diabetic myofibrils, a measurement of sulfhydryl content of the myofibrils was carried out (Figures 8 and 9). A time course of myofibrillar sulfhydryl group reactivity to

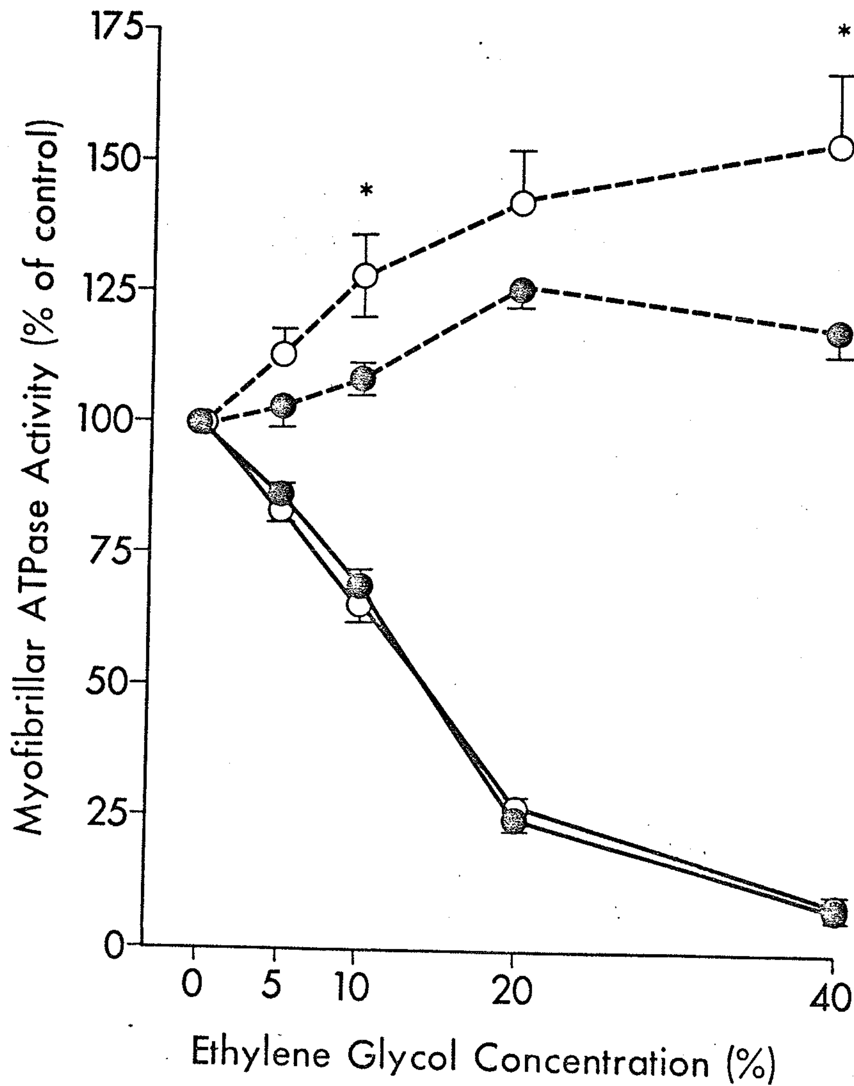

Figure 6. Effect of varying concentrations of ethylene glycol on cardiac myofibrillar basal (---) and Ca^{2+}-stimulated (——) ATPase activities in control (●) and diabetic (O) animals. Each value is expressed as percentage of respective control value in the absence of ethylene glycol. 100% control and experimental basal ATPase activities were 0.22 ± 0.01 and 0.16 ± 0.02 μmol Pi/mg/5 min, respectively. 100% control and experimental Ca^{2+}-stimulated ATPase activities were 0.77 ± 0.01 and 0.69 ± 0.03 μmol Pi/mg/5 min, respectively. * $P < 0.05$ vs. control values (n = 6).

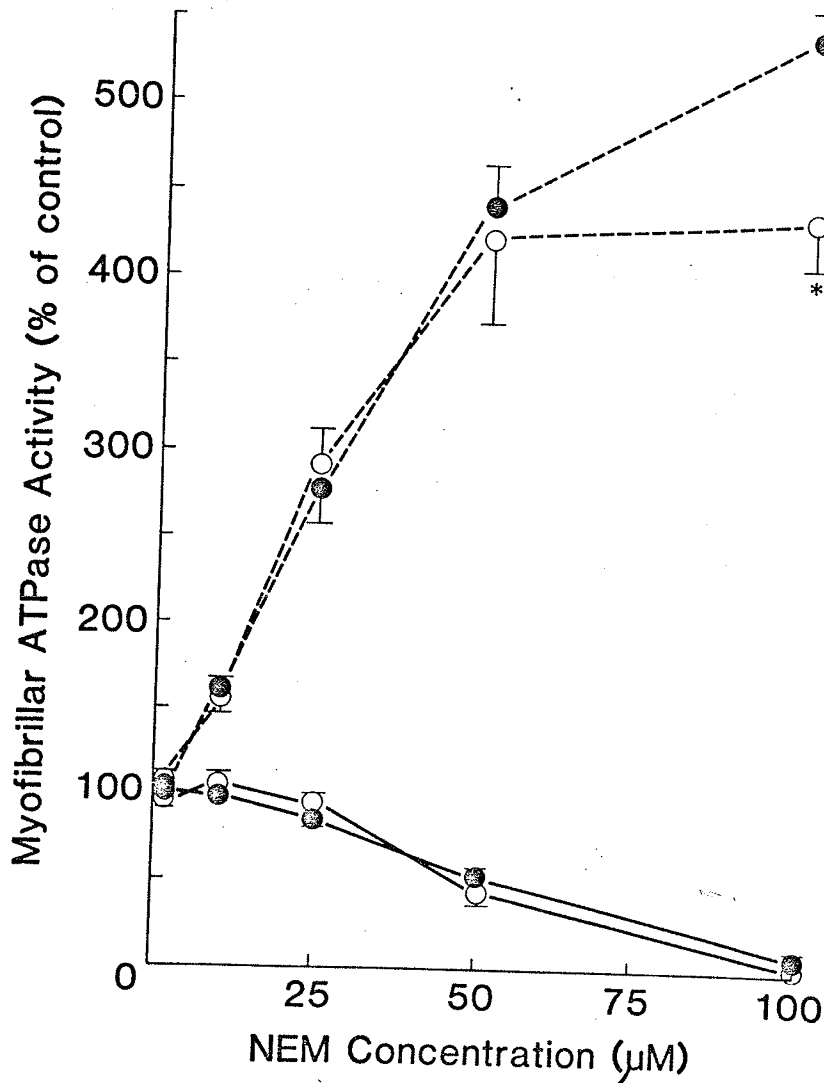

Figure 7. Effect of varying concentrations of N-ethylmaleimide (NEM) on cardiac myofibrillar basal (---) and Ca^{2+}-stimulated (——) ATPase activities in control (●) and diabetic (O) animals. Each value is expressed as a % of activity observed in the absence of NEM. * $P < 0.05$ vs. control (n = 6).

5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), another sulfhydryl reagent, was determined and the results are shown in Figure 8. Sulfhydryl content in diabetic rat heart myofibrils was found to be significantly lower in comparison to control preparations at all time points investigated. Insulin administration to the diabetic animals reversed this effect. DTNB concentrations were also varied and sulfhydryl group reactivity in myofibrillar samples examined (Figure 9). As was the case in the time-course study, sulfhydryl group content was significantly lower in diabetic rat heart myofibrils in comparison to control in the presence of all of the DTNB concentrations employed. Insulin treatment of the diabetic rats restored myofibrillar sulfhydryl reactivity to a level such that it did not differ from control values.

C. Alterations in Cardiac Sarcolemma in Diabetic Rats.

Enzyme activities associated with the sarcolemmal membrane were examined (Table 4) in order to characterize these preparations and determine the possible extent of cross-contamination by other subcellular organelles. The activities of all of the enzymes examined in the homogenate were statistically similar between control and diabetic groups. Sarcolemmal Mg^{2+}-ATPase activity was significantly elevated and 5'-nucleotidase activity was depressed in diabetic preparations in comparison to control. Patent Na^+, K^+-ATPase and patent ouabain-sensitive Na^+, K^+-ATPase activities in this fraction did not differ between the two groups. Ouabain-sensitive Na^+, K^+-ATPase and 5'-nucleotidase activities were enriched approximately 8-fold and 5-fold respectively in the sarcolemmal fractions from both groups suggesting that the relative purity of these samples was similar. In view of the belief that both Na^+, K^+-ATPase and 5'-nucleotidase are predominantly sarcolemmal in

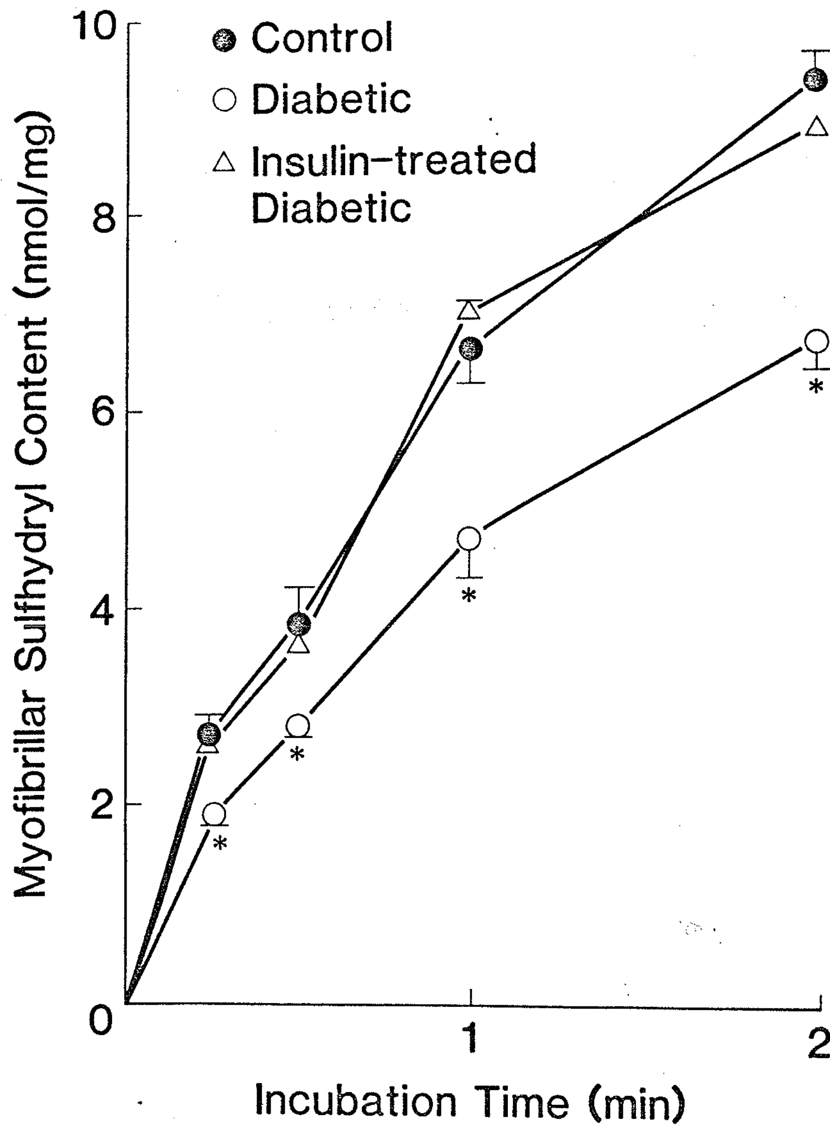

Figure 8. Time dependency of sulfhydryl group reactivity to 25 μM 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) in cardiac myofibrils from control, diabetic and insulin-treated diabetic rats. Values represent mean ± S.E. from 4 experiments. * $P < 0.05$ vs. control.

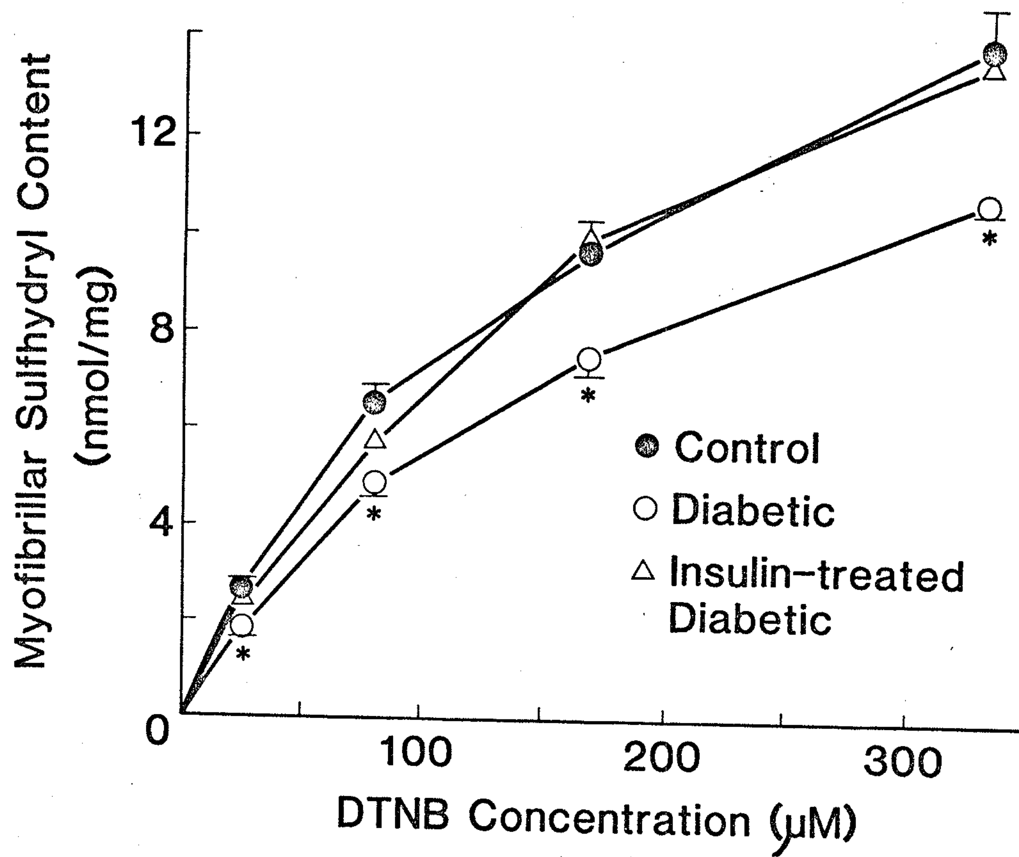

Figure 9. Sulfhydryl group reactivity in the presence of varying concentrations of 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) in cardiac myofibrils from control, diabetic and insulin-treated diabetic rats. The reaction time was 15 seconds. Each value represents the mean ± S.E. of 4 experiments. * $P < 0.05$ vs. control.

Table 4. Enzymatic activities in homogenate and sarcolemmal fractions isolated from ventricular tissue from control and diabetic rats.

Activity	Control			Diabetic		
	Homogenate	Sarcolemma	Purity Factor	Homogenate	Sarcolemma	Purity Factor
Na^+, K^+-ATPase	ND	17.80 ± 0.22	--	ND	18.72 ± 0.22	--
Ouabain-sensitive Na^+, K^+-ATPase	1.51 ± 0.24	12.21 ± 0.18	8.09	1.46 ± 0.12	11.41 ± 0.21	7.82
Mg^{2+}-ATPase	49.21 ± 0.30	50.56 ± 0.46	1.03	47.01 ± 2.47	62.12 ± 0.53*	1.32
5'-Nucleotidase	28.12 ± 1.59	145.8 ± 3.89	5.18	22.23 ± 1.01*	116.9 ± 7.70*	5.26
Cytochrome C oxidase	271.0 ± 34.9	151.6 ± 12.2	0.56	219.3 ± 35.6	108.3 ± 9.8	0.49
K^+-EDTA ATPase	15.43 ± 1.43	ND	0.00	14.67 ± 0.57	ND	0.00
Ca^{2+}-stimulated ATPase	5.09 ± 0.60	ND	0.00	2.99 ± 0.91	ND	0.00

Values represent means ± S.E. of 20 experiments for the sarcolemmal fractions and 5 experiments for the homogenate fraction. ATPase activity is expressed in μmol/Pi/mg/hr. Cytochrome C oxidase and 5'-nucleotidase activities are presented as nmol cytochrome oxidized/mg/min and nmol adenosine formed/mg/min, respectively. ND: non-detectable. *denotes significant difference from respective control value ($P < 0.05$).

origin (Bers, 1979; Dhalla et al, 1977; Schwartz et al, 1975), this data agrees well with that reported by others (Takeo et al, 1979; Matsukubo et al, 1981) that this membrane preparation is derived from the sarcolemma. A reduction in the purification of cytochrome C oxidase activity as well as indetectable K^{+}-EDTA ATPase and Ca^{2+}-stimulated ATPase activities in control and experimental sarcolemmal samples demonstrates that there is minimal contamination by mitochondrial, contractile protein and sarcoplasmic reticular fractions, respectively (Bers, 1979; Martin et al, 1982). In addition, since there were no differences between the two groups in the relative purification of these membranes from the homogenate, this would rule out any major artifact associated with the preparations employed here. SDS-gel electrophoretic separation of the sarcolemmal proteins revealed the appearance of no new proteins in the diabetic preparation in comparison to control (Figure 10) which would offer additional gross evidence of a similar degree of purity in these membrane fractions. However, the results in Figure 10 indicate an increase in a sarcolemmal protein identified as approximately 70,000 molecular weight in the diabetic preparations. This peak represented 2.3% of the total protein in control preparations whereas this value increased to 6.6% in sarcolemma from diabetic animals.

The sarcolemmal preparation employed in this study was found to bind calcium in the absence of ATP at both low and high affinity sites. Thus ATP-independent sarcolemmal calcium binding was examined in diabetic rat heart in the presence of both 0.05 and 1.25 mM calcium and was found to be significantly depressed (Table 5). This defect in sarcolemmal Ca^{2+} binding was found to be insulin-reversible. The control values for calcium binding with heart sarcolemma are in agreement with those reported elsewhere (Matsukubo et al, 1981; Philipson et al, 1980).

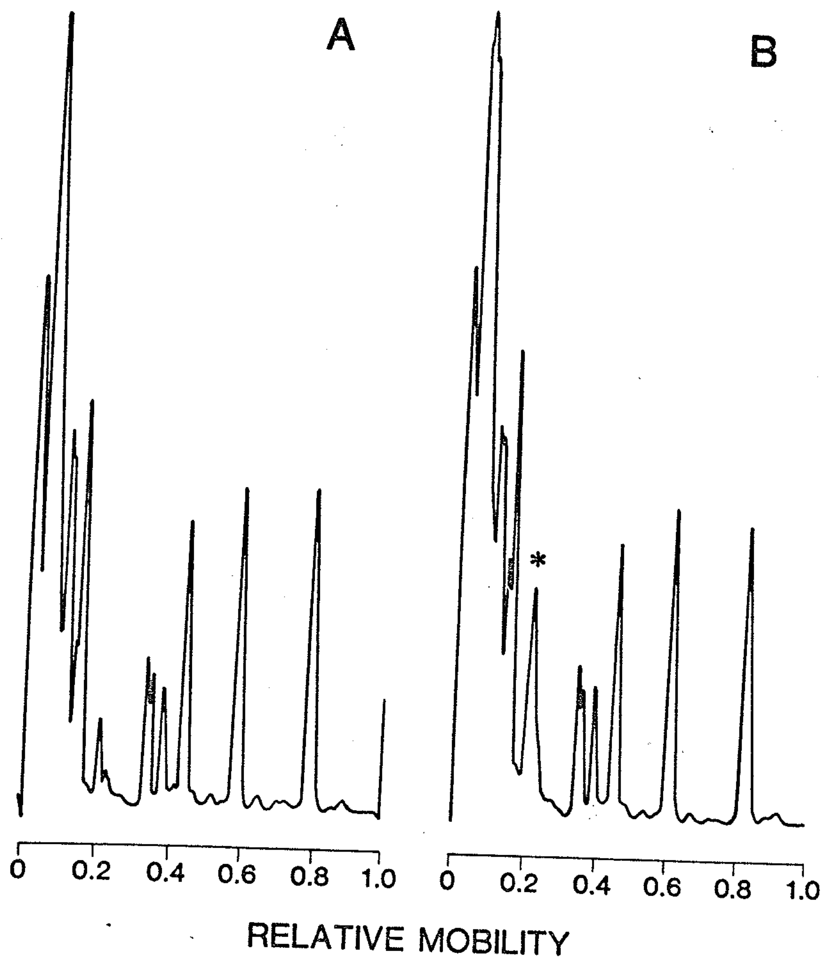

Figure 10. Densitometric scans of sarcolemmal protein separated by SDS polyacrylamide gel electrophoresis. Separation was run in a 10% acrylamide gel in the presence of appropriate standard molecular weights (Sigma). Sarcolemmal membrane protein was isolated from: A. Control rats and B. Diabetic rats.

* Denotes alteration in percentage composition of underlying peak in comparison to respective control protein peak.

Table 5. Sarcolemmal Ca^{2+} binding capacity in hearts from control, diabetic and insulin-treated diabetic rats.

	Control	Diabetic	Diabetic + Insulin
1.25 mM Ca^{2+} binding (nmol/mg)	193.38 ± 16.70	87.23* ± 4.00	160.74 ± 6.06
0.05 mM Ca^{2+} binding (nmol/mg)	15.99 ± 1.55	11.58* ± 0.07*	15.58 ± 1.19

Values represent mean ± S.E. of 4 experiments. * $P < 0.05$ vs control values.

The mechanism which may explain the observed depression in sarcolemmal calcium binding may reside in some alteration in those components of the sarcolemmal membrane which bind calcium. Since some phospholipids are known to have the ability to bind calcium (Philipson et al, 1980), the phospholipid composition of both control and diabetic rat heart sarcolemma was investigated (Table 6). Phospholipid values for control rat sarcolemma reported here are similar to those found elsewhere in the literature (Panagia et al, 1982; Owen et al, 1979). The concentration of lysophosphatidylcholine was increased and the levels of phosphatidylethanolamine and diphosphatidylglycerol were depressed in sarcolemma from diabetic rat heart. Other phospholipids were unaltered in the present study. Total cholesterol content was increased in sarcolemmal membranes from diabetic rat hearts, however total phospholipid content and the cholesterol : phospholipid ratio of the membranes did not differ between the two groups. The cholesterol : phospholipid ratio observed in the present study is similar to that reported elsewhere (Tibbits et al, 1981).

Another sarcolemmal calcium binding pool in the heart is reported to be associated with the sialic acid residues (Langer, 1978; Matsukubo et al, 1981; Takeo et al, 1980; Frank et al, 1977). Sialic acid content of myocardial sarcolemmal membranes was found to be significantly depressed in diabetic rat hearts (Table 7); this effect was insulin reversible. Control values for the sialic acid content are similar to those reported elsewhere in the literature (Matsukubo et al, 1981; Takeo et al, 1980; McConnaughey et al, 1979). The sensitivity of the sarcolemmal-bound Ca^{2+} to neuraminidase treatment was also examined. Neuraminidase is an enzyme which specifically cleaves the sialic acid residue from the membrane-bound glycoprotein (Langer, 1978; Frank et al, 1977). The highest concentration of neuraminidase employed in this study released

Table 6. Phospholipid and cholesterol composition of cardiac sarcolemmal membranes isolated from control and diabetic rats.

Lipid	Control	Diabetic
A. Amount of Phospholipid (% of total)		
Phosphatidylcholine	39.97 ± 3.51	40.03 ± 3.20
Lysophosphatidylcholine	0.52 ± 0.26	1.91 ± 0.27*
Phosphatidylethanolamine	39.04 ± 0.89	30.54 ± 2.20*
Sphingomyelin	5.28 ± 1.38	8.72 ± 1.34
Phosphatidylserine	5.90 ± 1.47	6.60 ± 1.80
Phosphatidylinositol	2.71 ± 0.50	3.27 ± 1.29
Diphosphatidylglycerol	9.07 ± 0.55	6.00 ± 1.12*
B. Total Phospholipid Content (nmol/mg)	187.65 ± 6.4	201.59 ± 18.3
C. Cholesterol Content (nmol/mg)	103.24 ± 2.0	125.31 ± 7.3*
D. Cholesterol: Phospholipid Ratio (M:M)	0.55 ± 0.01	0.64 ± 0.05

Results are expressed as a mean ± S.E. of 3-6 experiments. * Significant difference from control ($P < 0.05$).

Table 7. Sialic acid content, sensitivity of Ca^{2+} binding to neuraminidase and sialyltransferase activity in cardiac sarcolemmal membranes isolated from control, diabetic and insulin-treated diabetic rats.

	Sialic Acid Content (nmol/mg)	Neuraminidase-insensitive Ca^{2+} binding (%)		Sialyltransferase Activity (pmol/mg/hr)
		0.05 U/mg	0.5 U/mg	
Control	35.15 ± 2.77	78.44 ± 6.08	63.53 ± 9.14	1.49 ± 0.16
Diabetic	25.23 ± 1.36*	111.59 ± 8.69*	124.52 ± 34.67*	1.84 ± 0.16
Diabetic + Insulin	31.31 ± 1.99	- - -	- - -	- - -

Values represent mean ± S.E. of 4 experiments. Ca^{2+} binding data is expressed as a % of Ca^{2+} capacity of untreated membranes. Neuraminidase-insensitive Ca^{2+} binding refers to Ca^{2+} binding still present after neuraminidase treatment. * Indicates significant difference from control values ($P < 0.05$). - -: not determined.

approximately 50-60% of membrane bound sialic acid (Matsukubo et al, 1981; Takeo et al, 1980). As shown in Table 7, neuraminidase treatment of sarcolemmal membranes from diabetic rats did not decrease their calcium binding capacity whereas calcium binding by control membranes was reduced by up to 37%. Sialyltransferase activity was also determined and found to be unaffected by the diabetic condition of the animal (Table 7). The addition of 0.05 or 0.1 mM ATP to the incubation medium to inhibit any endogenous non-specific phosphatase activity had no significant effect on either control or diabetic sample sialyltransferase activity. It should be noted that sialyltransferase activity reported here is similar to that reported elsewhere in cardiac ghost plasma membranes (Bailey and Ma, 1980).

Ca^{2+}-dependent ATPase activity in the myocardial sarcolemmal vesicles was significantly elevated in the diabetic samples in comparison to control (Table 8). This effect was reversible upon daily insulin administration to the diabetic rats. Two cations, Mn^{2+} and Ni^{2+}, which are known to inhibit Ca^{2+}-dependent ATPase activity were examined with respect to their inhibitory effects on this activity in the three groups. Both Mn^{2+} (1 mM) and Ni^{2+} (1 mM) inhibited Ca^{2+} dependent ATPase activity in control, diabetic and insulin-treated diabetic sarcolemmal samples to a similar extent.

Further characterization of the abnormal Ca^{2+}-dependent ATPase activity was carried out in the presence of different Ca^{2+} or ATP concentrations (Table 9). Varying the ATP levels from 0.1 mM to 4 mM in the incubation medium revealed similar K_m values from Ca^{2+}-dependent ATPase activity in the control and diabetic groups (0.198 mM in control; 0.191 mM in diabetic) whereas the V_{max} value for diabetic preparations

Table 8. Ca^{2+}-dependent ATPase activity in sarcolemma from control, diabetic and insulin-treated diabetic rats.

	Control	Diabetic	Diabetic + Insulin
Ca^{2+}-dependent ATPase activity	56.4 ± 4.7	69.3 ± 3.3*	48.5 ± 6.1
Ca^{2+}-dependent ATPase activity in presence of 1 mM Mn^{2+}	45.2 ± 2.0	54.6 ± 2.1	38.8 ± 5.0
Ca^{2+}-dependent ATPase activity in presence of 1 mM Ni^{2+}	24.8 ± 1.0	28.8 ± 1.1	20.7 ± 3.1

Values represent mean ± S.E. of 4-8 samples. * $P < 0.05$ vs control.

Activities are expressed as μmol Pi/mg/hr.

Table 9. Kinetic characterization of Ca^{2+}-dependent ATPase activity in sarcolemma from control and diabetic rats.

	Control	Diabetic
K_m for ATP (mM)	0.198	0.191
V_{max} for ATP (μmol Pi/mg/hr)	54.9	65.8
K_a for Ca^{2+} (mM)	0.794	0.795
V_{max} for Ca^{2+} (μmol Pi/mg/hr)	56.8	70.8

Values are presented as the mean of at least 3 experiments. ATP and $CaCl_2$ concentrations in the reaction medium were varied from 0.1 mM to 4 mM.

(65.8 μmol Pi/mg/hr) differed from the control value (54.9 μmol Pi/mg/hr). Similarly, varying the Ca^{2+} concentration from 0.1 mM to 4 mM revealed changes in V_{max} (56.8 μmol Pi/mg/hr in control; 70.8 μM Pi/mg/hr in diabetic) but no alterations in K_a values (0.794 mM in control; 0.795 mM in diabetic) were observed.

Control membranes used in this study have been characterized as being predominantly right-side out oriented (Takeo et al, 1979; Matsukubo et al, 1981). Since the active site of Na^+, K^+-ATPase activity is located on the inner or cytoplasmic face of the sarcolemmal membrane (Schwartz et al, 1975), it is possible that the total Na^+, K^+-ATPase activity was not fully expressed in the experiments presented in Table 4. Therefore, in order to increase membrane permeability and expose latent Na^+, K^+-ATPase activity, the sarcolemmal fractions from both control and experimental groups were treated with a detergent, deoxycholate (Panagia et al, 1982) and alamethicin, an antibiotic ionophore with known channel-creating capacity (Jones et al, 1980). Both agents have been shown to increase Na^+, K^+-ATPase activity in membranous preparations by exposing more catalytic sites for enzymatic reaction (Schwartz et al, 1975; Jones et al, 1980; Panagia et al, 1982). As shown in Fig. 11, sarcolemmal Mg^{2+}-dependent ATPase activity was unaffected by deoxycholate (DOC) treatment of the membranes in comparison to the untreated normal values in control, diabetic and insulin-treated diabetic preparations. However, DOC treatment significantly increased sarcolemmal Na^+, K^+-ATPase activity in control and insulin-treated diabetic samples but did not significantly alter this activity in diabetic samples. Similarly, ouabain-sensitive Na^+, K^+-ATPase activity was enhanced in control preparations after DOC exposure while this activity in sarcolemmal samples from diabetic animals was actually depressed after DOC. The mechanism responsible for this

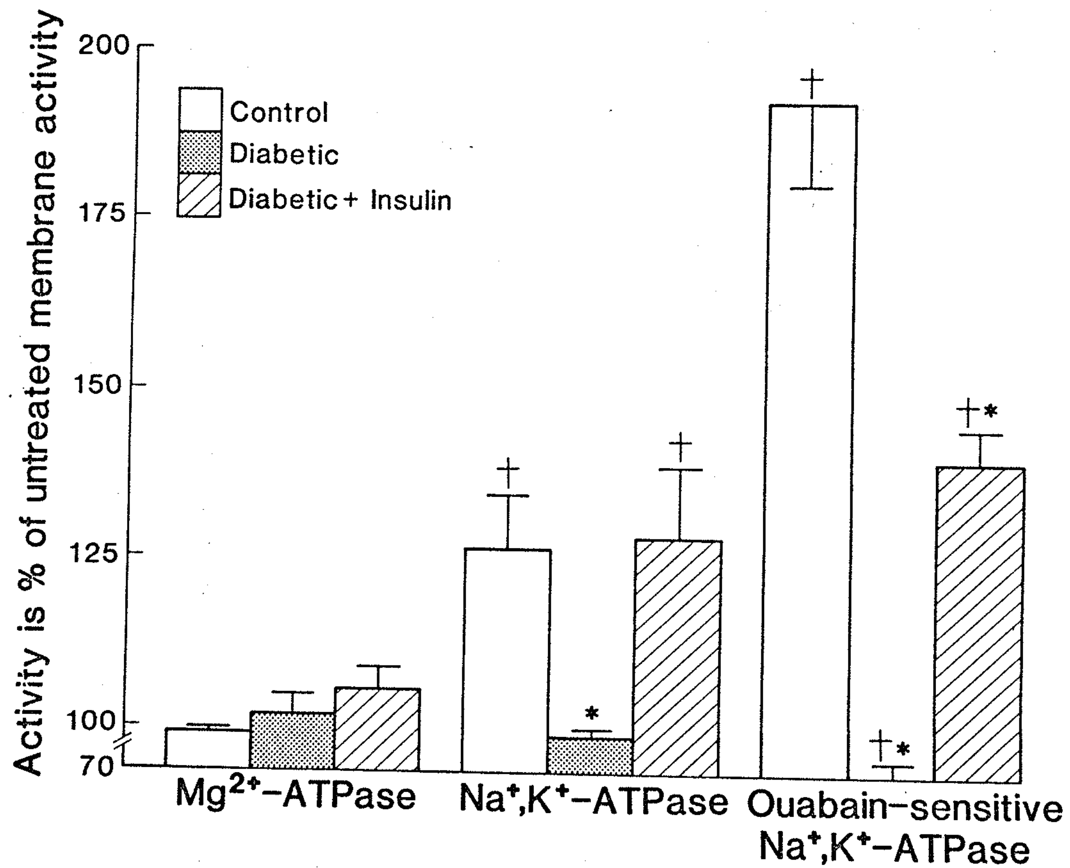

Figure 11. Effect of deoxycholate treatment of sarcolemmal membranes on Mg^{2+}-ATPase, Na^{+}, K^{+}-ATPase and ouabain sensitive Na^{+}, K^{+}-ATPase activities. Each value is expressed as a % of respective, untreated membrane enzyme activity. + Significant effect of deoxycholate treatment ($P < 0.05$). * Value differs from respective control ($P < 0.05$). Values are means ± S.E. of 4-6 experiments.

depression is unclear, however, it differed significantly from latent ouabain-sensitive Na^+, K^+-ATPase activity in control preparations. In insulin-treated diabetic samples, sarcolemmal ouabain-sensitive Na^+, K^+-ATPase activity was significantly increased after DOC treatment, and, although this response represented a significant recovery in comparison to diabetic animals, it was still lower than control values.

These results, therefore, clearly reveal a defect in latent Na^+, K^+-ATPase activities in diabetic rat preparations after deoxycholate treatment. This defect appears to be largely reversible after insulin prophylaxis has been instituted in vivo to maintain glucose homeostasis. In addition, preliminary data have indicated that latent and patent Na^+, K^+-ATPase activities were similar in control and diabetic animals two weeks after streptozotocin injection. This would suggest therefore, that the defect in latent Na^+, K^+-ATPase activities was associated with the chronic diabetic condition and appears to be related with the hypo-insulinemic status of these animals.

The concentration of DOC employed in this study was carefully chosen (Panagia et al, 1982) and appeared to succeed in being low enough to avoid any gross, non-specific membrane detergent effects as evidenced by the lack of effect of this agent on Mg^{2+}-ATPase activity in all fractions. However, in order to determine if this was solely due to some property inherent in this particular sarcolemmal membrane as isolated by the LiBr procedure, a different isolation procedure of the sarcolemmal membrane fraction in the heart was employed. The sucrose density procedure of Philipson and Nishimoto (1981) was chosen because of its high purity and detailed characterization (Bers, 1979). As shown in Table 10, patent ouabain-sensitive Na^+, K^+-ATPase activity in control and diabetic rat

Table 10. Effect of deoxycholate treatment of sarcolemma isolated by the sucrose-density gradient method on ouabain-sensitive Na^+, K^+-ATPase activity in control and diabetic rat hearts.

Group	Ouabain-sensitive Na^+, K^+-ATPase activity (μmol Pi/mg/hr)	
	Patent	Latent
Control	18.91 ± 0.53	25.98 ± 1.00$^+$
Diabetic	17.57 ± 0.90	17.25 ± 1.47*

Values represent mean ± S.E. of 4 experiments. ATPase activity determined in deoxycholate-treated membranes was termed as latent ATPase activity whereas that determined in the absence of pre-treatment with this agent was described as patent ATPase activity. Sucrose-density gradient isolation procedure of the sarcolemmal membranes was carried out as described by Philipson and Nishimoto (1981). $^+$ Significant effect of drug treatment ($P < 0.05$). * Significant difference from control.

membranes were 18.91 ± 0.53 μmol Pi/mg/hr and 17.57 ± 0.90 μmol Pi/mg/hr, respectively, values similar to that reported elsewhere (Tibbits et al, 1981) using this technique for the isolation of sarcolemmal membranes from rat heart. Diabetic preparations exhibited reduced ($P < 0.05$) latent ouabain-sensitive Na^+, K^+-ATPase activities in comparison to control values. As well, DOC treatment stimulated patent activity in control preparations to a greater degree than was observed in the experimental samples. It is pointed out that latent ouabain-sensitive Na^+, K^+-ATPase activities in control heart membranes obtained by the sucrose density gradient and hypotonic shock-LiBr treatment methods were about 26 and 24 μmoles Pi/mg/hr, respectively. Thus the purification factors for both of these membrane preparations on the basis of latent ouabain-sensitive Na^+, K^+-ATPase activities were similar.

In order to determine the specificity of the response of sarcolemmal Na^+, K^+-ATPase activity to a membrane perturbing agent, alamethicin (ALA) was also employed in the present study. Although the exact mechanism of its action is unclear, its capacity to disrupt membrane permeability barriers may not resemble that of a detergent-like effect (Jones et al, 1980). Therefore, sarcolemmal Mg^{2+}- and Na^+, K^+-ATPase activities were examined in control and diabetic preparations after ALA pretreatment. As shown in Table 11, ALA decreased sarcolemmal Mg^{2+}-ATPase activity to a similar extent in both preparations (19.5% and 21.1% in control and diabetic samples, respectively). The response of Na^+, K^+-ATPase activities to ALA was characteristically similar to the response after DOC treatment. Na^+, K^+-ATPase and ouabain-sensitive Na^+, K^+-ATPase activities were enhanced in control membranes by 36 ± 5% and 18.5 ± 2.5%, respectively ($P < 0.05$) but diabetic membrane preparations did not significantly alter these activities in response to ALA exposure.

Table 11. Mg^{2+}-ATPase, Na^{+}, K-ATPase and K^{+}-paranitrophenyl phosphatase activities in control and diabetic rat sarcolemma before and after alamethicin treatment.

Enzyme Activity	Patent Activities		Latent Activities	
	Control	Diabetic	Control	Diabetic
Mg^{2+}-ATPase (μmol Pi/mg/hr)	51.42 ± 0.72	58.58 ± 0.35*	$41.75 \pm 3.41^{+}$	$46.28 \pm 3.71^{+}$
Na^{+}, K^{+}-ATPase (μmol Pi/mg/hr)	17.94 ± 0.88	18.32 ± 1.23	$24.28 \pm 1.03^{+}$	19.37 ± 1.36*
Ouabain-sensitive Na^{+}, K^{+}-ATPase (μmol Pi/mg/hr)	11.85 ± 0.32	10.35 ± 0.42	$13.83 \pm 0.42^{+}$	10.14 ± 0.06*
K^{+}-pNPPase (μmol phenolate/mg/hr)	2.08 ± 0.12	1.40 ± 0.03*	$3.14 \pm 0.09^{+}$	$2.34 \pm 0.12^{+*}$
Ouabain-sensitive K^{+}-pNPPase (μmol phenolate/mg/hr)	0.65 ± 0.06	0.39 ± 0.07*	$1.43 \pm 0.05^{+}$	$1.08 \pm 0.13^{+*}$

Values are means ± S.E. of 4 experiments. K^{+}-pNPPase: K^{+}-stimulated p-nitrophenyl phosphatase. Patent activity refers to that activity observed in membranes which were not pretreated with ALA. Latent activity refers to that activity expressed after the membranes were pretreated with ALA. + Significant effect of drug treatment ($P < 0.05$). * Value differs significantly from respective, similarly treated control value ($P < 0.05$).

In addition, K^+-stimulated pNPPase activity was studied in the present set of experiments in order to answer two questions: firstly, it was necessary to confirm a report of decreased K^+-pNPPase activity in a diabetic cardiac myocyte preparation (Onji and Liu, 1980), and secondly, alamethicin treatment of this enzyme could reveal more information concerning the sensitivity of the Na^+, K^+-ATPase enzyme system to alamethicin. It is possible that the lower latent Na^+, K^+-ATPase activities expressed in the diabetic preparations after ALA treatment were simply due to a reduced sensitivity of these membranes to these agents. As shown in Table 11, K^+-pNPPase activity was depressed in the cardiac sarcolemmal fraction isolated from diabetic rats. Ouabain-sensitive K^+-pNPPase was similarly depressed in diabetic animals. Both K^+-pNPPase and ouabain-sensitive K^+-pNPPase activities in control and diabetic preparations were equally sensitive to ALA treatment. Control K^+-pNPPase activity increased 151 ± 12% whereas diabetic K^+-pNPPase activity increased 167 ± 7% after ALA treatment ($P < 0.05$). Ouabain-sensitive K^+-pNPPase activity in control and diabetic sarcolemmal samples increased 220 ± 40 and 276 ± 21%, respectively, after ALA treatment ($P < 0.05$). However, absolute values for latent K^+-pNPPase and latent ouabain-sensitive K^+-pNPPase activities were still significantly lower in diabetic than control preparations (Table 11).

Alterations in membrane lipid composition are known to affect membrane electrical characteristics and enzyme activities (Alivisatos et al, 1977). Since total cholesterol content was increased in sarcolemmal membranes from diabetic rat hearts (Table 6), it is possible that this lipid abnormality may be responsible in part for the altered sarcolemmal ATPase activities. In order to examine the possible effects of the altered membrane cholesterol levels in cardiac sarcolemmal from diabetic animals,

filipin, a polyene antibiotic agent, was employed. Filipin is known to bind preferentially to cholesterol residues and has been shown to alter cardiac adenylate cyclase activity by this mechanism (Bittman, 1978; Lad et al, 1979). As depicted in Figure 12, treatment with varying filipin concentrations altered Mg^{2+}-dependent ATPase activity to a similar extent in both groups. However, sarcolemmal Na^{+}, K^{+}-ATPase activity in diabetic preparations was found to be more resistant to filipin administration; this achieved statistical significance at a sarcolemma : filipin ratio of 10 : 1.

D. Cardiac Mitochondrial Function in Diabetic Rats.

In order to determine if the mitochondrial vesicles which were isolated from the three groups, control, diabetic and insulin-treated diabetic, may be subject to differential contamination, a biochemical characterization of mitochondrial purity was undertaken (Table 12). The sensitivity of the mitochondrial fraction to 5 mM NaN_3 was extremely high with regard to Ca^{2+} uptake, Ca^{2+} binding and Mg^{2+}-ATPase activities. Sodium azide inhibited these activities completely or up to 80%. This is strongly supportive evidence that this preparation was predominantly mitochondrial in nature (Robertson and Boyer, 1955). In addition, the sensitivity of these activities to azide was similar among all three of the groups which would indicate that these preparations were of the same relative purity. The lack of Ca^{2+}-stimulated ATPase activity in the presence of 10-100 μM $CaCl_2$ is also supportive of the contention that there is negligible myofibrillar and sarcoplasmic reticular contamination. Ouabain-sensitive Na^{+}, K^{+}-ATPase activity was purified approximately 0.6 fold from the homogenate fraction. There was no significant difference in this activity in the mitochondrial preparations

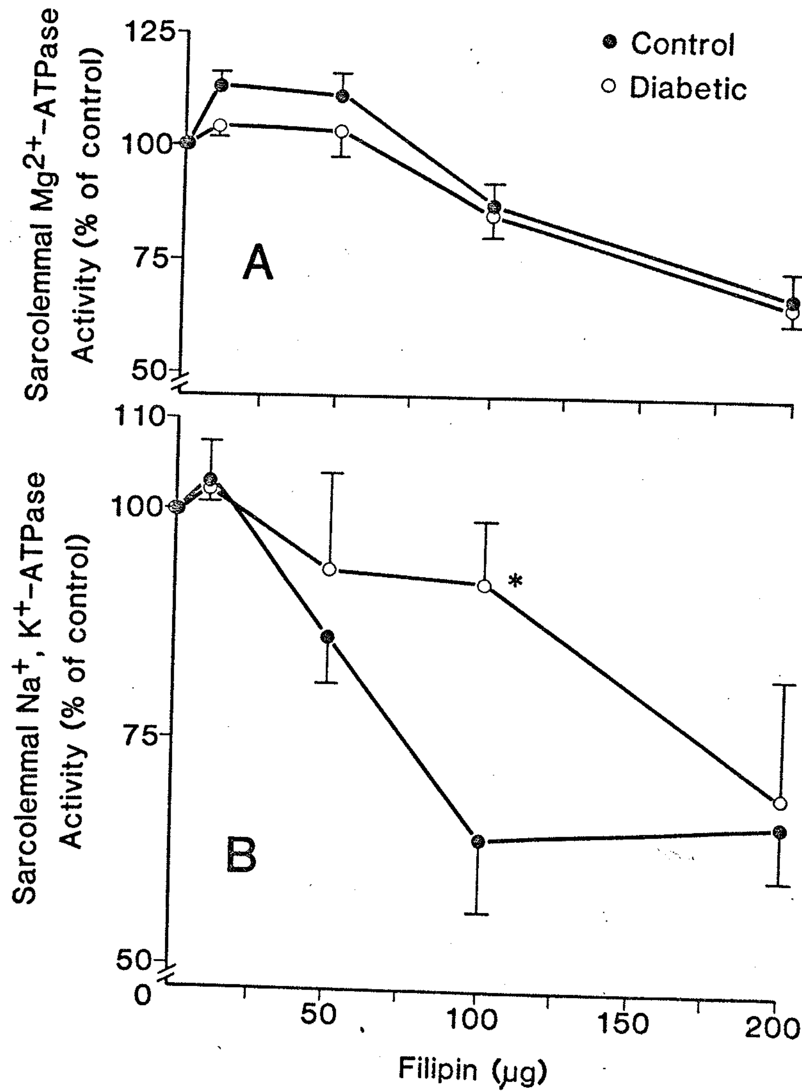

Figure 12. Cardiac sarcolemmal (A) Mg^{2+}-ATPase and (B) Na^+, K^+-ATPase activities after filipin treatment. Values are presented as a % of untreated membrane enzyme activity. * Value differs from respective control values ($P < 0.05$). Values are means ± S.E. of 5 experiments.

Table 12. Biochemical characterization of the mitochondrial fraction isolated from hearts from control, diabetic and insulin-treated diabetic animals.

	Control	Diabetic	Diabetic + Insulin
Azide-sensitive Ca^{2+} uptake	100 ± 0.0	100 ± 0.0	100 + 0.0
Azide sensitive Ca^{2+} binding	95 ± 2.2	97 ± 5.0	93 ± 6.4
Azide sensitive Mg^{2+} ATPase	82 ± 7.8	88 ± 8.0	87 ± 9.1
Ca^{2+}-stimulated ATPase	ND	ND	ND
Ouabain-sensitive Na^{+},K^{+}-ATPase	1.00 ± 0.25	0.83 ± 0.41	0.88 ± 0.09
K^{+}-EDTA ATPase	ND	ND	ND

Values represent mean ± S.E. of 4 experiments. Azide sensitive activity is that activity inhibited by 5 mM NaN_3 and presented as a % of the activity observed in the absence of NaN_3. Ouabain-sensitive Na^{+}, K^{+}-ATPase activity is expressed in μmol Pi/mg/hr. ND: not detectable.

from among the three groups. On the basis of these findings, therefore, there appears to be minimal myofibrillar, sarcolemmal or sarcoplasmic reticular contamination of this mitochondrial preparation and the fractions from the three groups exhibited the same relative purity characteristics.

Mitochondrial oxidative phosphorylation was examined in preparations from control, diabetic and insulin-treated diabetic groups (Table 13). Values reported in the present study concerning parameters of oxidative phosphorylation in control animals are similar to those found by other investigators (Vaghy et al, 1981; Van Jaarsveld and Lochner, 1982). Using glutamate as a substrate, a significant depression in State 3 respiration and the oxidative phosphorylation rate (OPR) were observed in diabetic samples in comparison to control values. This effect was only partially reversed after insulin treatment as OPR values remained significantly depressed in the insulin-treated diabetic group in comparison to control values. In accord with these results, State 3 respiration and OPR values were depressed in diabetic preparations in the presence of pyruvate-maleate as a substrate. In addition, RCI values were lower in the diabetic samples than in control preparations. Insulin therapy of the diabetic animals reversed these effects only with regard to the RCI values.

Since mitochondrial Mg^{2+}-dependent ATPase activity has been previously correlated to State 3 respiration (Rouslin and Millard, 1981), it was considered to be of interest to examine this activity in the present study. Mg^{2+}-ATPase activity was significantly depressed in diabetic samples in comparison to control across a number of incubation times (Figure 13). Insulin prophylaxis returned this activity towards control values, however, these values still differed from control activities. The control Mg^{2+}-ATPase activity is similar to that reported elsewhere

Table 13. Mitochondrial oxidative phosphorylation in hearts from control, diabetic and insulin-treated diabetic animals.

	ADP/0	State 3	State 4	RCI	OPR
1. Glutamate					
a) Control	2.92 ± 0.20	46.32 ± 1.99	4.67 ± 0.55	10.52 ± 1.3	138.8 ± 13.1
b) Diabetic	2.48 ± 0.25	38.88 ± 2.56*	5.81 ± 1.14	7.55 ± 1.3	98.3 ± 11.7*
c) Diabetic + Insulin	2.40 ± 0.35	41.85 ± 1.94	5.05 ± 0.33	8.33 ± 0.3	98.3 ± 9.3*
2. Pyruvate-Malate					
a) Control	3.09 ± 0.13	51.25 ± 3.1	9.98 ± 1.07	5.39 ± 0.53	159.3 ± 12.0
b) Diabetic	2.58 ± 0.33	39.57 ± 1.5*	9.74 ± 0.06	4.10 ± 0.17*	102.3 ± 13.0*
c) Diabetic + Insulin	2.87 ± 0.44	37.76 ± 3.4*	7.03 ± 0.77	5.52 ± 0.65	110.8 ± 23.7

Values represent the mean ± S.E. of 4-6 experiments. States 3 and 4 respiration are expressed as natoms 0/mg/min. RCI: respiratory control index; ADP/0: ratio of nmol ADP phosphorylated per natoms O_2 consumed; OPR: oxidative phosphorylation rate; OPR values are expressed in nmoles ADP phosphorylated/mg/min and are calculated by State 3 x ADP/0 ratio.

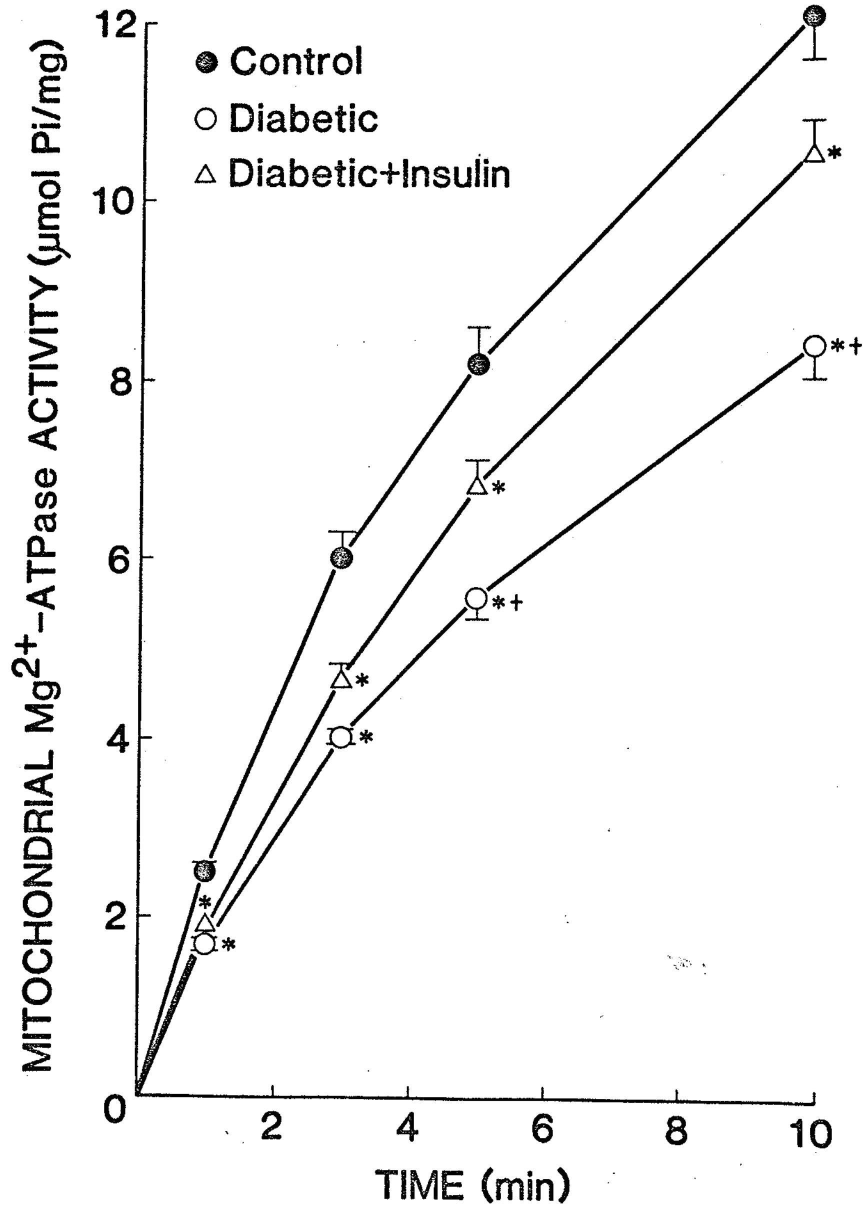

Figure 13. Mitochondrial Mg^{2+}-ATPase activity in hearts from control, diabetic and insulin-treated diabetic rats. Values represent mean ± S.E. of 5 experiments. * $P < 0.05$ vs control values. + $P < 0.05$ vs control and diabetic + insulin groups.

(Lee and Dhalla, 1976; Rouslin and Millard, 1981).

Mitochondrial Ca^{2+} uptake activity was examined in the presence of 100 µM $^{45}CaCl_2$ over a number of incubation times in Figure 14. Ca^{2+} uptake was significantly lower in the diabetic samples than the other 2 groups at all times examined. In order to determine if such accumulation was concentration dependent, Ca^{2+} uptake by mitochondria was studied at calcium concentrations from 10-100 µM (Table 14). Only at the higher calcium levels (50 & 100 µM) was a depression in mitochondrial uptake capacity observed in the diabetic samples. Again this effect was reversed upon chronic insulin administration in the diabetic animals.

Mitochondrial Ca^{2+} binding was also examined in the present study across various times of incubation (Table 15). There was no significant difference in the Ca^{2+} binding capacity of mitochondria isolated from control or diabetic hearts. In accord with these findings, varying the calcium concentration did not reveal any difference in mitochondrial Ca^{2+} binding between the two groups (Table 15). The values observed for mitochondrial Ca^{2+} uptake and binding in control preparations in this study are similar to values reported previously elsewhere (Lee and Dhalla, 1976).

In order to evalute the possibility that structural alterations in the mitochondria may contribute to the altered function exhibited by the diabetic preparations, gel electrophoretic separation of the mitochondrial proteins was carried out (Figure 15). There were no observable differences in mitochondrial protein composition in hearts from control, diabetic or insulin-treated diabetic rats.

Membrane lipid composition was also examined (Table 16). A significant increase in phosphatidylinositol and a decrease in lysophosphatidylcholine composition of the mitochondrial samples from diabetic rats were

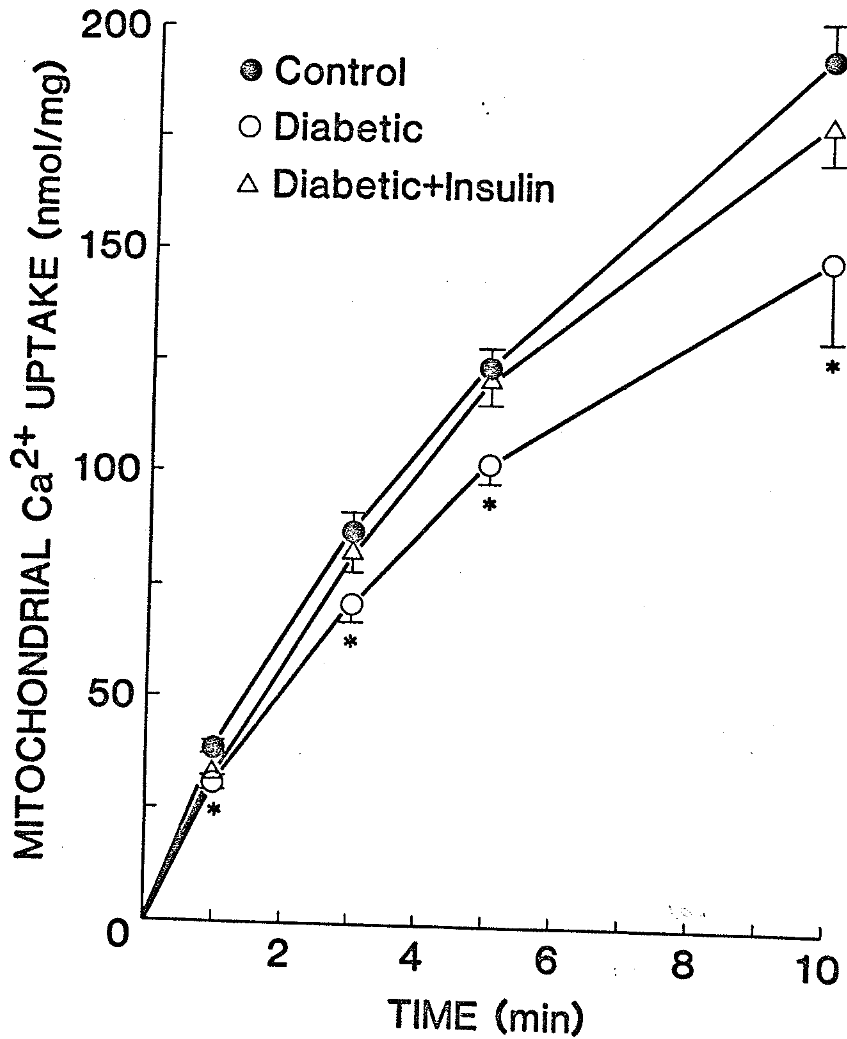

Figure 14. Mitochondrial Ca^{2+} uptake examined over various times of incubation in control, diabetic and insulin-treated diabetic samples. Values represent mean ± S.E. of 5 experiments. * $P < 0.05$ vs. control values. 100 μM $^{45}CaCl_2$ was present in the incubation medium.

Table 14. Cardiac mitochondrial calcium uptake activity in control, diabetic and insulin-treated diabetic rats.

Group	Calcium Concentration (μM)			
	10	25	50	100
Control	6.76 ± 0.36	23.91 ± 0.95	63.80 ± 3.06	137.65 ± 5.81
Diabetic	6.63 ± 0.67	19.99 ± 1.59	51.05* ± 1.60	113.18* ± 4.84
Diabetic + Insulin	8.23 ± 0.35	24.18 ± 1.27	58.53 ± 2.28	123.72 ± 7.42

Values represent the mean ± S.E.M. of 4-5 experiments. Activity is expressed as nmol/mg/5 min. * $P < 0.05$ vs. control value.

Table 15. Cardiac mitochondrial calcium binding in control and diabetic rats at different times of incubation and in the presence of varying calcium concentrations.

	Control	Diabetic	P level
A. Incubation Time			
1 minute	18.4 ± 4.0	16.0 ± 2.0	> 0.05
3 minutes	36.2 ± 5.8	35.5 ± 1.5	> 0.05
5 minutes	51.41 ± 5.3	50.7 ± 4.8	> 0.05
10 minutes	71.3 ± 5.3	74.0 ± 6.2	> 0.05
B. Calcium Concentration			
10 μM	5.68 ± 0.14	5.26 ± 0.31	> 0.05
25 μM	14.09 ± 1.3	13.02 ± 1.0	> 0.05
50 μM	29.43 ± 2.6	27.98 ± 1.7	> 0.05
100 μM	51.41 ± 5.3	50.73 ± 4.8	> 0.05

Values represent the mean ± S.E.M. of five animals. Activity is expressed in nmol Ca^{2+}/mg in A. and nmol Ca^{2+}/mg/5 min in B. In A. there was 100 μM Ca^{2+} present in the incubation medium.

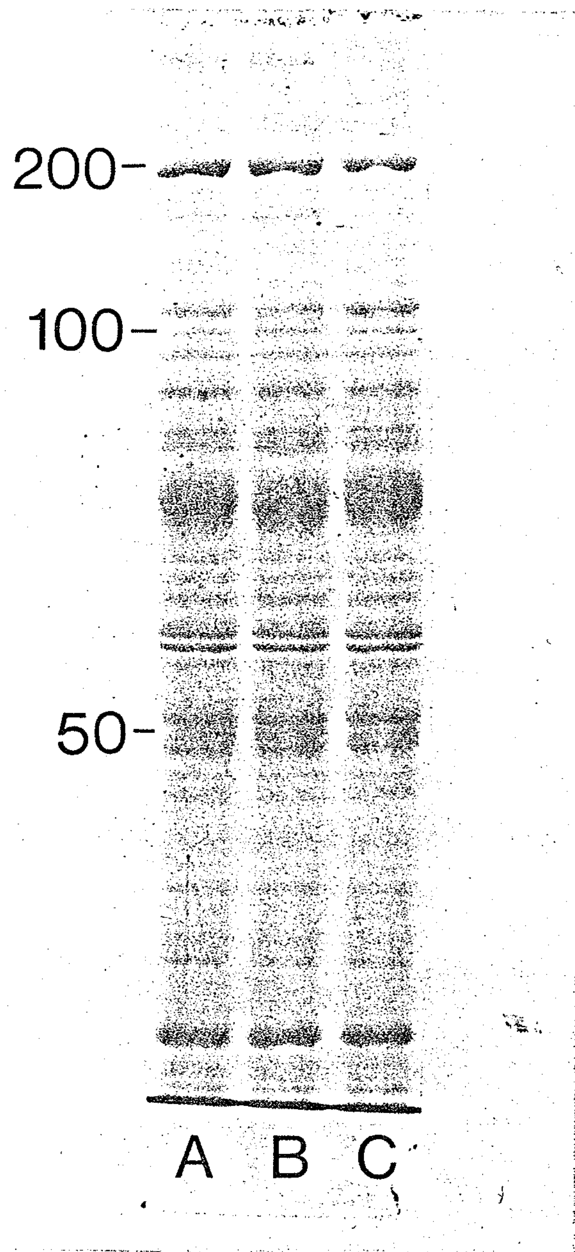

Figure 15. Representative gels from SDS electrophoretic separation of mitochondrial protein samples from the control (A), diabetic (B) and insulin-treated diabetic (C) groups. Electrophoresis was carried out as described in Methods and molecular weights presented were obtained from running of known standard molecular weights (Sigma).

Table 16. Mitochondrial lipid profile in hearts from control and diabetic rats.

	Amount of Phospholipid (% of total)	
	Control	Diabetic
A. Phospholipids		
Phosphatidylcholine	37.83 ± 1.90	40.73 ± 4.05
Lysophosphatidylcholine	3.85 ± 0.22	2.73 ± 0.26*
Phosphatidylethanolamine	26.03 ± 0.93	21.08 ± 1.72
Sphingomyelin	5.23 ± 0.17	3.90 ± 0.53
Phosphatidylserine	13.33 ± 0.81	11.15 ± 0.69
Phosphatidylinositol	4.70 ± 0.78	8.80 ± 1.76*
Diphosphatidylglycerol	8.15 ± 1.27	10.08 ± 1.28
Phosphatidic acid	0.85 ± 0.44	1.60 ± 0.15
Total Phospholipid Content (nmol Pi/mg)	223.7 ± 7.35	246.9 ± 14.31
B. Cholesterol Content (nmol/mg)	33.54 ± 2.10	30.05 ± 3.62
C. Cholesterol: Phospholipid Ratio (molar)	0.149 ± 0.02	0.122 ± 0.01

Results are expressed as the mean ± S.E. of 4 experiments. * $P < 0.05$ vs control value.

the only alterations in membrane lipid observed in the present study. Mitochondrial cholesterol content observed in the present study is similar to that reported previously (Rouslin et al, 1982)

V. DISCUSSION

A. General Features of Streptozotocin-Induced Diabetes in Rats.

Intravenous injection of streptozotocin at the dosage employed in this study resulted in highly elevated blood glucose levels concomitant with depressed insulin concentrations. This hyperglycemic condition is secondary to the hypoinsulinemic state. Streptozotocin is a toxic drug which is relatively specific for the β-cells of the pancreas (Arison et al, 1967; Junod et al, 1967). Within approximately 24 hours after injection, animals become chronically hypoinsulinemic due to necrosis of the pancreatic β-cells which synthesize and secrete insulin (Agarwal, 1980). The hypothyroid and hypercholesterolemic conditions which were found to accompany diabetes in the rats in the present study have been reported by other investigators in both human and experimental diabetes (Penpargkul et al, 1980; Turlapaty et al, 1980; Saunders et al, 1978; New et al, 1963) and therefore they were not conditions peculiar to or side-effects of the streptozotocin injection per se. The diabetic animals in the present study also exhibited little body or heart growth after streptozotocin injection. These morphometric alterations occurred in spite of increased food and water intake (Hofteizer and Carpenter, 1973) and probably result from dehydration and the abnormally high protein and fat catabolism which is maintained during diabetes (Oakley, 1968). The elevated ventricular: body weight ratio observed in the diabetic animals has been reported elsewhere (Penpargkul et al, 1980; Vadlamudi et al, 1982) and although no direct measurement was undertaken here, histological work on hearts from diabetic rats have suggested the hearts to be hypertrophic at a cellular level (Onishi et al, 1981).

B. Chronic Diabetes and Myofibrillar Function.

The results of the present study demonstrate a significant depression in the myofibrillar basal and Ca^{2+}-stimulated ATPase activities in myocardium from diabetic rats. These results extend earlier observations regarding depressed myosin and actomyosin ATPase activities in cardiac tissue from diabetic animals (Dillman, 1980; Malhotra et al, 1981; Fein et al, 1981). Further, the depression in myofibrillar Ca^{2+}-stimulated ATPase activity shown here occurs across a physiological range of Ca^{2+} concentrations. This depression in Ca^{2+}-stimulated ATPase activity does not appear to be due to changes in the dependence of diabetic myofibrils on free calcium concentrations. The possibility that these alterations in myofibrillar ATPase activities were caused by a direct toxic action of streptozotocin appears unlikely. In this regard, it is pointed out that abnormal heart function in streptozotocin-induced diabetic animals was not seen when the diabetogenic effect of streptozotocin injection was blocked by 3-0-methyl glucose (Fein et al, 1980). The observation that the defects in myofibrillar ATPase activities were insulin-reversible is more conclusive evidence that these effects were not associated with streptozotocin-induced cardiotoxicity but instead closely related to the diabetic state per se. However, it remains to be established whether similar myofibrillar abnormalities occur in spontaneously diabetic and other drug-induced diabetic animal models which are known to exhibit similar heart dysfunction (Regan et al, 1974; Giacomelli and Weiner, 1979).

The significance of this defect in myofibrillar ATPase activities, particularly that of Ca^{2+}-stimulated ATPase activity, lies in its close relationship with force generation in the heart. Elevated ATPase activity in myocardial contractile proteins has been associated with increases in contractile force (Wilkerson et al, 1971; Giusti et al, 1978) and,

conversely, depressed ATPase activity has been associated with diseased and failing hearts (Dowell, 1976; Alpert and Gordon, 1962). Most importantly, the biochemical activation of myofibrillar ATPase activity has been correlated to mechanical activation of contraction in cardiac tissue (Solaro et al, 1974). The depression in myofibrillar ATPase activity in diabetic rat heart may, therefore, be closely related to the contractile dysfunction.

Since varying KCl concentrations have been reported to alter the ATPase activity of contractile proteins by disrupting protein structure, differing responses to KCl by two separate preparations has been interpreted to suggest fundamental differences in the contractile protein structure (Warren et al, 1966). Accordingly, in the present study the increased sensitivity of myofibrillar basal and Ca^{2+}-stimulated ATPase activity to varying KCl concentrations in diabetic preparations may indicate structurally different forms of the protein. These results support those of Dillmann (1980) which show an alteration in the composition of myosin isoenzymes in the diabetic myocardium. A shift in the myosin isoenzyme composition from the "fast" V_1 form to the "slow" V_3 form has been previously reported in diabetic rat heart (Dillmann, 1980). and since the myosin isoenzymic distribution has been closely correlated with the speed of myocardial contraction (Schwartz et al, 1981), this provides further biochemical clues to the nature of the defect in heart contractile function during diabetes.

The possibility that more obvious, gross structural changes in the myofibrillar protein from diabetic rat heart may participate in the cardiomyopathy was also plausible on the basis of existing data. Two preliminary studies (Shiozaki et al, 1979; Maeno et al, 1981) have observed

quantitative alterations in the actin or myosin components of the contractile proteins isolated from diabetic animal and human hearts. The results of the present study obtained by SDS-gel electrophoretic separation of the myofibrillar proteins demonstrated no significant change in these proteins during diabetes. These results confirm those of Malhotra et al (1981) which showed no alteration in actin or myosin composition in samples from control and streptozotocin-induced diabetic rat heats. The reason for the discrepancy in results between the studies is unclear especially in light of the preliminary nature of the former two studies, however, differences in experimental protocol or species variation may be responsible.

In addition to the subtle structural changes uncovered by the KCl experiments above, several other factors including conformation changes at or near the active site of ATPase activity can be seen to account for the observed depression in myofibrillar ATPase activity in diabetic myocardium. This view regarding conformational changes in diabetic myofibrillar protein is substantiated by the interaction of myofibrils with ethylene glycol, which has been shown to alter the enzymatic activity of contractile proteins by effecting conformational changes through a modification of sulfhydryl groups, histidine groups or possibly rupturing the hydrophobic region around the active site of ATPase activity on the myofibril (Kaldor, 1968). The possibility that the sulfhydryl groups may be modified during diabetes is particularly attractive since the modification of sulfhydryl groups has been shown to lead to local conformational changes in the myosin molecule and the inactivation of myosin ATPase activity (Sekine et al, 1962). Since the site responsible for the conformational alterations indicated by the ethylene glycol experiments was still relatively vague, NEM treatment of the cardiac myo-

fibrils was also carried out. Since NEM modifies sulfhydryl groups (Sekine et al, 1962), an alteration in ATPase activity in the presence of NEM should indicate alterations in protein conformation via sulfhydryl group modification (Yazaki and Raben, 1974). Myofibrillar Mg^{2+}-ATPase activity from control and diabetic rat hearts responded differently to high NEM concentrations in the present study which would suggest thiol group modification was present in the experimental preparations. This contention was confirmed by the experiments employing DTNB to measure sulfhydryl group content in the myofibrillar samples. Sulfhydryl content of myofibrils from diabetic rats were depressed in comparison to control and insulin-treated diabetic preparations at all DTNB concentrations and time points examined in the reaction. It is important to point out that since the DTNB reaction did not exhibit saturation - type kinetics , this data cannot be interpreted to give any information concerning total sulfhydryl content. Rather, this data can only yield information concerning sulfhydryl group reactivity with DTNB and is, therefore, more useful with regard to elucidating the availability of the sulfhydryl groups to DTNB (Habeeb, 1972). Thus this data would be supportive of a specific alteration in myofibrillar conformation at the thiol group sites and it is possible that this modification in the myofibril may be responsible for the depression in the ATPase activity in diabetic rat hearts.

In summary, therefore, the defective utilization of high energy phosphates by the myofibrillar proteins observed in the present study may contribute to the reported depression in contractile function of the diabetic heart (Fein et al, 1980; Penpargkul et al, 1980). Similar conclusions have been drawn from observations with several types of failing hearts (Scheuer and Bhan, 1979). This, however, does not exclude the possibility

that altered enzymatic activities of different membrane systems may also contribute to the diabetic cardiodepression.

C. Chronic Diabetes and Sarcolemmal Function.

The results of the present study describe major alterations in the ability of the myocardial sarcolemma from diabetic rats to handle calcium. On the basis of the biochemical data and those using gel electrophoretic protein separation, the results do not appear to be confounded by any differences in the relative purity of these fractions. In addition, on the basis of studies which have employed spontaneously diabetic animals, blockade of the diabetogenic action of streptozotocin, and regular insulin therapy to streptozotocin-induced diabetic animals prior to sacrifice (Regan et al, 1974; Penpargkul et al, 1980; Fein et al, 1981), it is very improbable that streptozotocin itself has any cardiotoxic effect either at a subcellular or tissue level. Furthermore, most of the effects observed in diabetic animals were reversible by in vivo insulin administration making it highly unlikely that they were the result of direct streptozotocin action. Therefore, the alterations in cardiac sarcolemma appear to be associated with the diabetic condition.

The defect in cardiac sarcolemmal calcium binding at both high and low affinity binding sites may be of critical importance to the function of the diabetic rat heart. Calcium bound to the sarcolemmal pool has been integrally related to force generation in the heart (Bers et al, 1981; Dhalla et al, 1982). Accordingly, a pathophysiological alteration in the capacity of this superficial calcium pool may severely hamper the mechanical performance of the heart. A similar conclusion has been reached in various other types of cardiomyopathies (Dhalla et al, 1978, and 1982). Therefore, this sarcolemmal defect may to some extent

contribute to the diabetic cardiodepression reported previously (Penpargkul et al, 1980; Fein et al, 1981). Work with isolated heart preparations confirms the presence of an abnormal response by diabetic animals to extracellular calcium (Bielefeld and Boshell, 1980; Gotzsche, 1981).

The mechanism responsible for this decrease in calcium binding appears to reside in the low sialic acid content of the diabetic membranes. Such a depression in Ca^{2+} binding may be partly due to a reduction in the neuraminidase-sensitive sialic acid residues because neuraminidase treatment of the diabetic preparation, unlike the control membranes, failed to decrease the Ca^{2+}-binding activity. This decrease in membrane sialic acid content during diabetes appears to be a generalized phenomenon in the body since lowered contents of sialic acid have been reported in connective tissue (Berenson et al, 1972), erythrocytes (Baba et al, 1978) and glomerular basement membrane (Westberg and Michael, 1973) from human and animal models of diabetes. In addition, the decrease in diphosphatidylglycerol content in the diabetic membranes may also contribute to the decrease in Ca^{2+} binding since this acidic phospholipid has been shown to bind substantial quantities of Ca^{2+} (Philipson et al, 1980). This Ca^{2+} pool is believed to be involved in contractile force regulation (Philipson et al, 1980).

The absence of a decrease in any of the sarcolemmal proteins from the diabetic samples would provide gross preliminary evidence that protein alterations were not involved in the defect in Ca^{2+} binding. However, it may be of interest to note that the 70,000 dalton peak which was elevated in content in the diabetic preparations corresponds to the estimated molecular weight of the sarcolemmal Ca^{2+}-dependent ATPase protein (Tuana and Dhalla, 1982). Sarcolemmal Ca^{2+}-dependent ATPase

has been suggested to function in the normal myocardium as a membrane gating protein regulating calcium entry into the cell (Dhalla et al, 1982). Its activity has been correlated closely with sarcolemmal calcium binding capacity and cardiac contractile force development (Dhalla et al, 1982). The increased activity of the sarcolemmal Ca^{2+}-dependent ATPase in the diabetic rat heart may represent an uncoupling of that relationship. This pathophysiological situation may therefore involve some type of compensatory response on the part of the Ca^{2+}-dependent ATPase enzyme in order to balance the depression in sarcolemmal bound calcium available for the contractile process. If Ca^{2+}-dependent ATPase in the cell membrane is taken to be a receptor for calcium (Zukin and Koshland, 1976; Anand-Srivastava et al, 1982), then it appears that depressed calcium binding in diabetic sarcolemmal preparations cannot be explained on the basis of changes in such sites in the plasma membrane. At any rate, elevated activity of Ca^{2+}-dependent ATPase in diabetic preparations provides further evidence that Ca^{2+}-related functions of sarcolemmal membrane are abnormal in diabetic rat heart.

The reason which may account for the depression in sarcolemmal sialic acid in the diabetic cardiomyopathy is unclear since sialyltransferase activity was not decreased in diabetic preparations. Sialyltransferase, the enzyme responsible for the attachment of sialic acid onto the membrane, has been reported to be depressed in renal tissue from diabetic mice (Bardos et al, 1980) and heart muscle from cardiomyopathic hamsters (Bailey and Ma, 1980). It is possible that the defect in diabetic cardiomyopathy may instead lie elsewhere along the pathway of ultimate attachment of the sialic acid onto the proper position on the glycoprotein (Spiro, 1969) rather than at the level of sialyltransferase. In this regard, a recent study has demonstrated a defect in pyrimidine

nucleotide metabolism in diabetic rat heart (Gertz and Haugaard, 1979). Since pyrimidine nucleotides function as cofactors in the synthesis of sialic acid (Gertz and Haugaard, 1979), it is possible that the reduction in sarcolemmal sialic acid content shown in the present study may be a result of this alteration.

Although changes in the basic phospholipids are not likely to contribute to the depression in Ca^{2+} binding demonstrated in the diabetic preparations, these alterations may be of some importance to cellular integrity. Lysophosphatidylcholine accumulation in the myocardium has been associated with electrophysiological abnormalities (Katz and Messineo, 1981) and, in fact, alterations in electrical activity in the diabetic rabbit heart have been reported previously (Senges et al, 1980). Phosphatidylethanolamine levels were also decreased in cardiac sarcolemmal preparations from diabetic rats in the present investigation and degradation of this phospholipid has been demonstrated to be associated with defects in sarcolemmal permeability in the myocardium (Chien et al, 1981). This observation is particularly interesting since removal of sarcolemmal sialic acid residues in the heart has also been closely associated with an increase in membrane permeability (Frank et al, 1977; Langer, 1978). The increase in sarcolemmal cholesterol content is also significant in this regard. Exposure of cultured heart cells to moderate cholesterol concentrations has been found to increase permeability characteristics of lysosomal and mitochondrial membranes (Wenzel et al, 1975). Increases in sodium and calcium permeability have been suggested to occur in hypercholesterolemic states (Pfeiffer et al, 1978; Peterson et al, 1979). The enhanced sarcolemmal cholesterol content observed in diabetic rats in the present study, which is probably related to the elevated plasma cholesterol levels, may therefore act to increase

membrane permeability in the hearts of these animals as well. It is possible therefore, that the decreases in membrane sialic acid and phosphatidylethanolamine content and the increase in cholesterol content observed in the present investigation may concertedly act to alter permeability characteristics of the cell membrane from diabetic rat hearts. The decreased binding of Ca^{2+} to the sarcolemma from diabetic rats may itself also change permeability characteristics by altering physical properties of the membrane (Gordon et al, 1978). In this regard, reports of increases in vascular tissue permeability have been documented early in the diabetic disease process (Joyner et al, 1981). This hypothesis of a change in the integrity of the cardiac sarcolemmal membrane would agree well with published accounts of altered cation contents in the myocardium of diabetic animals (Regan et al, 1981; Nagase et al, 1981), however, it would need more direct analysis to determine its presence conclusively.

This hypothesis regarding an alteration in sarcolemmal integrity stimulated an extensive examination of sarcolemmal Na^+, K^+-ATPase activity in hearts from diabetic rats. The results observed in the present study indicated no change in patent Na^+, K^+-ATPase activity but a depression in K^+-pNPPase activity in sarcolemma from diabetic rat heart. The latter observation confirms earlier work using diabetic dog myocyte preparations (Onji and Liu, 1980). Patent Na^+, K^+-ATPase activity in skeletal muscle sarcolemma from diabetic rats has also been found to be unaltered (Olson et al, 1981). Since the Na^+, K^+-ATPase and K^+-pNPPase enzymatic activities are believed to be coupled (Schwartz et al, 1975), then these findings could indicate either a specific lesion in the diabetic Na^+, K^+-ATPase enzyme system, or some alteration in the active catalytic site of Na^+, K^+-ATPase with respect to the

accessibility of the substrate. This active site is known to be on the inner face of the sarcolemmal membrane (Schwartz et al, 1975), therefore, in an effort to fully expose Na^+, K^+-ATPase enzyme activity, membranes were treated with ALA and DOC. Both these agents are known to increase membrane permeability (Jones et al, 1980; Schwartz et al, 1975) and are considered to increase enzymatic activity primarily by disrupting membrane barriers and revealing latent catalytic sites (Jones et al, 1980; Schwartz et al, 1975). They are thought to have little capacity to alter enzymatic activity through any intrinsic modifying effect on the catalytic activity of the enzyme (Jones et al, 1980; Schwartz et al, 1975). Latent Na^+, K^+-ATPase activities exposed after treatment with these agents were significantly lower in the diabetic samples. It would appear, therefore, that the depression in K^+-pNPPase activity is accompanied by a similar defect in the Na^+, K^+-ATPase enzyme. The present observation does not seem to be an artifact associated with this particular membrane preparation obtained by the hypotonic shock-LiBr method since it was confirmed through the use of another sarcolemmal membrane isolated by the sucrose density gradient procedure. As well, this defect in Na^+, K^+-ATPase activity was largely insulin reversible which suggests that this lesion is associated with the disease condition directly and further argues persuasively against the contention that streptozotocin may exert specific cardiotoxic effects. It is possible that the sarcolemmal membranes prepared from the diabetic animals were not sensitive to the membrane perturbing agents and therefore latent Na^+, K^+-ATPase activity was not fully expressed. However, this hypothesis appears unlikely since both Mg^{2+}-ATPase and K^+-pNPPase activities were significantly altered by alamethicin treatment to a similar extent in both diabetic and control preparations. Furthermore, we employed two membrane perturbing agents

which may alter permeability through different mechanisms (Jones et al, 1980). In any event, the results of the present study agree well with an observation of depressed ouabain-sensitive ^{86}Rb uptake in ventricular slices from diabetic animals (Ku and Sellers, 1982).

Another interesting interpretation of the results obtained with the use of the membrane perturbing agents may involve an alteration in the permeability characteristics of the diabetic rat heart sarcolemmal membrane. Since DOC and ALA treatment did not increase Na^+, K^+-ATPase activities in the diabetic preparations, it could be assumed that latent activity was already being expressed in the absence of these agents. It is possible that these membranes were already "leaky" while control membranes which responded to ALA and DOC treatment demonstrated intact membrane barriers. Vesicles isolated by the sucrose gradient method showed a similar trend in response to DOC treatment such that control activity was stimulated to a greater extent than that seen in the diabetic samples (Table 10). This hypothesis would agree well with that proposed earlier with regard to the data on sarcolemmal composition. However, we cannot exclude the possibility that membranes from diabetic rat heart are oriented in such a way that some of the catalytic sites for Na^+, K^+-ATPase activity, which are hidden in the control preparations, become fully exposed upon isolation and are thus insensitive to treatment with ALA and DOC.

The mechanisms responsible for the alteration of sarcolemmal Na^+, K^+-ATPase function in hearts from diabetic animals may be of a complex nature. Clearly, the depressed Na^+, K^+-ATPase activity appears to be a consequence of one or all of the many metabolic changes associated with insulin-deficiency in this experimental model since this defect was largely reversible upon treatment of the diabetic animals with

insulin. The possibility that the conditions of ketosis, restricted weight gain and hypothyroidism which are associated with the diabetic disease (Fein et al, 1981; Penpargkul et al, 1980) could contribute to these sarcolemmal lesions cannot be excluded on the basis of the existing information. However, it should be emphasized that studies which have normalized plasma thyroid hormone levels in diabetic animals or compared diabetic animals to pair-fed control animals have failed to demonstrate a complete reversal of cardiac dysfunction or abnormalities in cardiac contractile protein integrity (Fein et al, 1981; Penpargkul et al, 1980). Indeed, the depression of sarcolemmal Na^+, K^+-ATPase activity is not limited to the chronic diabetic condition since this defect has been reported in different experimental models of heart disease (Dhalla et al, 1978).

One avenue which was explored in the present study involved the participation of membrane lipids in the defect in Na^+, K^+-ATPase activity. Alterations in the membrane lipid content are known to affect membrane enzymatic activities (Alivisatos et al, 1977). The results of the present study indicated an increase in membrane cholesterol in diabetic rat heart sarcolemma. Whether changes in cholesterol composition interfere with enzymatic activity in the cardiac sarcolemmal membranes was more closely investigated in the present study through the use of filipin, a polyene antibiotic agent which interacts primarily with membrane cholesterol residues (Bittmann, 1978). Filipin treatment depressed the sarcolemmal ATPase activities; this inhibitory effect is similar to that seen in cardiac adenylate cyclase experiments (Lad et al, 1979). Filipin treatment of the cardiac sarcolemmal membranes from diabetic rats demonstrated that the Na^+, K^+-ATPase activity responds differently to cholesterol modification, suggesting cholesterol may indeed exert some complex

effect on this activity in membranes from diabetic animals. This would agree well with another investigation which reported elevated sarcolemmal cholesterol concentrations were associated with depressed Na^+, K^+-ATPase activities in rats fed a cholesterol-enriched diet (Moffat and Dhalla, 1982). The present experiment, however, does not exclude the possibility that other factors may participate in the defect in Na^+, K^+-ATPase activity in diabetic rat hearts. In this regard, it is pointed out that increased levels of long chain acyl carnitine which have been demonstrated to exist in the diabetic rat myocardium (Feuvray et al, 1979) may also inhibit Na^+, K^+-ATPase activity (Wood et al, 1977) under diabetic conditions. The increased sarcolemmal concentration of lysophosphatidylcholine in diabetic preparations observed in the present study may also affect the Na^+, K^+-ATPase activity since Karli et al (1979) have observed that increased levels of this lipid can inhibit Na^+, K^+-ATPase activity.

The present study indicated that sarcolemmal Mg^{2+}-ATPase activity was increased whereas 5'-nucleotidase activity was decreased in diabetic myocardium. The observed increase in Mg^{2+}-ATPase activity in diabetic heart sarcolemmal membrane is particularly noteworthy since Mg^{2+}-ATPase activities in sarcoplasmic reticular and myofibrillar preparations from diabetic rat heart were decreased (Penpargkul et al, 1981; present study). This would suggest that contamination by other subcellular organelles in this membrane preparation was minimal in both control and experimental groups. This contention was confirmed by marker enzyme characterization which found no differences in mitochondrial, sarcoplasmic reticular or myofibrillar contamination of the sarcolemmal fractions obtained from control and diabetic animals on the basis of cytochrome C oxidase, cal-

cium-stimulated ATPase and K^+-EDTA ATPase activities, respectively. The depression in 5'-nucleotidase activity observed in the present study was unexpected in light of studies examining aspects of coronary patency during chronic experimental diabetes in rats. Coronary arteries appear absent of atherosclerotic development (Waber et al, 1981) and have enhanced coronary flow rates (Stam and Hulsmann, 1977; Penpargkul et al, 1980) in the streptozotocin-induced diabetic rat model. Since 5'-nucleotidase activity is responsible for the production of adenosine, a potent vasodilatory substance (Belloni, 1979), the depression in cardiac 5'-nucleotidase activity in the diabetic rat in the presence of elevated coronary flow rates represents a curious anomaly. It appears likely that the role of adenosine in coronary function has become severely altered during diabetes. This contention is supported by a recent study by Downing et al (1982) which found adenosine could increase coronary sinus flow in control lambs but had no effect on this parameter in diabetic lambs. It is important to note, though, that the coronary blood flow is regulated by many substances and factors other than adenosine (Belloni, 1979) and some evidence has been reported that changes in other vasodilatory substances may occur during diabetes (Stam and Hulsmann, 1977).

In summary, the observed alterations in membrane ion binding, enzyme activities and membrane composition can be interpreted to suggest some lesion in the sarcolemmal integrity in the chronically diabetic animal. This defect may have important significance in the expression of features of the diabetic cardiomyopathy. In particular, significant changes in membrane permeability and specific enzyme activities could influence cellular ionic homeostasis. Such alterations in myocardial

ionic composition may effect changes in cellular metabolism and structure (Dhalla et al, 1978; 1982) which can be conceived to be associated with the development of the diabetic cardiomyopathy.

D. Chronic Diabetes and Mitochondrial Function.

The present investigation demonstrated a generalized depression in mitochondrial respiratory function in the heart during the chronic diabetic state in the rat. This observation correlates well with electron microscopic evidence of swollen mitochondria with damaged cristae formation in hearts from chronically diabetic rats (Tarach, 1976; Giacomelli and Weiner, 1979; Onishi et al, 1981). Furthermore, this data supports the earlier contention of Haugaard and Haugaard (1964) who suggested oxidative phosphorylation capacity may be decreased in heart homogenates from diabetic rats. The present data is also in accord with that found earlier for mitochondrial respiratory activity 24-48 hours after alloxan injection (Kerbey et al, 1976; Puckett and Reddy, 1979). However, the relevance of the results from these earlier studies with respect to the diabetic condition is limited by two factors. Firstly, although these results have been commonly associated with the diabetic condition, examination of myocardial metabolism so soon after injection of the diabetogenic agent may instead reflect drastic changes in glycemic homeostasis rather than a relatively stable hyperglycemic condition. Five to 6 hours after injection of alloxan is an initial hyperglycemic response which is followed by an extended, seriously hypoglycemic stage for the next 18 hours (Stauffacher and Renold, 1971). Approximately 24-32 hours after injection, a hyperglycemic, hypoinsulinemic condition presents itself. It is doubtful that any conclusions obtained from studies carried out so soon after injection of the diabetogenic agent can hold much relevance

to the diabetic state per se. The present study, however, has largely eliminated the influence of this variable on the results by examining cardiac parameters eight weeks after injection. This represents nearly 60 days of stable, chronically elevated blood glucose levels. Secondly, these earlier investigations may have limited the significance of their findings by employing alloxan as the diabetogenic agent. Alloxan has been shown to significantly alter mitochondrial function in vitro (Nelson and Boquist, 1982). The possibility, therefore, exists that the previous conclusions may be tempered by the direct toxic action of alloxan on mitochondrial function. Streptozotocin, however, appears to have no observable effect on mitochondrial function in vitro (Nelson and Boquist, 1982). It appears reasonable to conclude, therefore, on the basis of a lack of cardiotoxic action by streptozotocin and the observation that many alterations found in the present study were at least partially insulin-reversible, that the depressed mitochondrial function demonstrated in this study was closely associated with the chronically diabetic state. Differential contamination of the control and experimental mitochondrial samples is also unlikely to have occurred. Neither control nor diabetic preparations exhibited Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase activity which strongly suggests myofibrillar, sarcolemmal, and sarcoplasmic reticular contamination was minimal. Furthermore, the Ca^{2+} transporting activities of mitochondria from all groups were highly sensitive to sodium azide, a response which is again suggestive that contamination of this preparation was negligible and similar between groups.

It is more difficult to establish what factors within the diabetic condition may be responsible for the lesion. Large vessel disease can

be ruled out in the streptozotocin-diabetic model (Waber et al, 1981), however, microvascular alterations in the heart may be present (Factor et al, 1980). This may induce local ischemic periods and since ischemia has been demonstrated to elicit major complications in mitochondrial function (Sordahl, 1979), it is possible that limitations in microvascular perfusion of the heart could have altered mitochondrial integrity in the present investigation. However, a myriad of metabolic and hormonal disturbances which accompany the diabetic condition cannot be dismissed as causally related factors on the basis of the results found in the present investigation. For example, the hypothyroid status of the streptozotocin-induced diabetic animals may also be a contributive factor. Depressions in State 3 and 4 respiration in mitochondria from hearts of hypothyroid rats have been reported (Nishiki et al, 1978). A similar depression in State 3 respiration has been observed in the present study. However, diabetic rats exhibited normal State 4 respiratory activity and a depression in RCI which would suggest that although the hypothyroid condition may participate in the mitochondrial dysfunction, certain features of mitochondrial function appear to be altered specifically in the diabetic condition. Plasma hypercholesterolemia does not appear to have influenced mitochondrial cholesterol composition and thus it would appear unlikely to be directly involved in the lesion in mitochondrial activities. The significance of the changes in phosphatidylinositol and lysophosphatidylcholine are unclear at present. Gross alterations in protein content within the mitochondria may be ruled out on the basis of the data obtained by protein separation by gel electrophoresis. It is conceivable, however, that more subtle alterations or a reorganization of mitochondrial composition may have occurred.

The depression in mitochondrial function is of obvious significance to the working of the heart in the diabetic animal. A defect in mitochondrial respiratory activity may eventually lead to a fall in cellular levels of high energy phosphates. Lowered levels of ATP have been reported in hearts from diabetic animals (Allison et al, 1976; Miller, 1979). Since low tissue levels of ATP are known to be closely associated with the necrotic process (Jennings et al, 1978), the depression in mitochondrial respiratory function in the diabetic myocardium may have important implications with regard to cell viability. In support of this, evidence has been reported suggestive of abnormally large necrotic damage in myocardium of diabetic humans (Nicod et al, 1982). The depression in Mg^{2+}-ATPase activity further supports the presence of a defect in respiratory function since Rouslin and Millard (1981) have maintained a close relationship between the two mitochondrial parameters. The impairment in mitochondrial Ca^{2+} uptake capacity observed in the diabetic samples is similar to that found in various models of cardiac dysfunction and failure. Indeed, accompanying depressions in mitochondrial Ca^{2+} uptake capacity and respiratory activities have been observed in ischemic hearts, failing human hearts, cardiomyopathic rabbits and animals given chronic dosages of alcohol (see Dhalla et al, 1978 for a review). Mitochondrial Ca^{2+} transport was also found to be diminished in rat hearts made to fail due to substrate deficiency (Muir et al, 1970).

In summary, this study has demonstrated the presence of impaired mitochondrial Mg^{2+}-ATPase, calcium uptake and respiratory activities in chronically diabetic rat heart. This data may provide, therefore, further information toward the elucidation of the metabolic basis for the diabetic cardiomyopathy.

E. An Overview of the Subcellular Basis of the Diabetic Cardiomyopathy.

On the basis of the results obtained from this investigation and other studies, it is possible to propose a general hypothesis to explain the mechanisms responsible for the diabetic cardiomyopathy from a cellular perspective (Figure 16). Since cardiac contractility and the viability of the heart as a whole is essentially determined by the concerted function of the various organelles comprising the myocardium (Dhalla et al, 1977; Dhalla et al, 1982), it becomes a rational progression that membrane and contractile protein integrity during diabetes be investigated.

The primary defect in the diabetic model employed in this study is chronic insulin deficiency and therefore, it is the ultimate cause of all subsequent lesions in the body. This is essentially true with regard to the diabetic cardiomyopathy. Contractile dysfunction, histological or subcellular alterations associated with the diabetic state have been demonstrated to be reversed by in vivo insulin administration in the present study and in many other investigations (Baandrup et al, 1981; Ku and Sellers, 1982; Murphy et al, 1981; Peckett and Reddy, 1979; Fein et al, 1981; Ganguly et al, 1983; Tahiliani et al, 1983).

Chronic insulin deficiency leads to elevated plasma lipid concentrations, presumably because of the antilipolytic effect of insulin (Newsholme, 1977). Indeed, Newsholme (1977) has proposed a controversial theory that the antilipolytic action of insulin is more important in controlling blood glucose levels than its capacity to directly stimulate membrane glucose transport. In any event, there is evidence which supports the presence of significantly elevated levels of cholesterol and triglycerides in plasma from streptozotocin-induced diabetic rats (Turlapaty et al, 1980; Waber et al, 1981) and moderately elevated levels of free

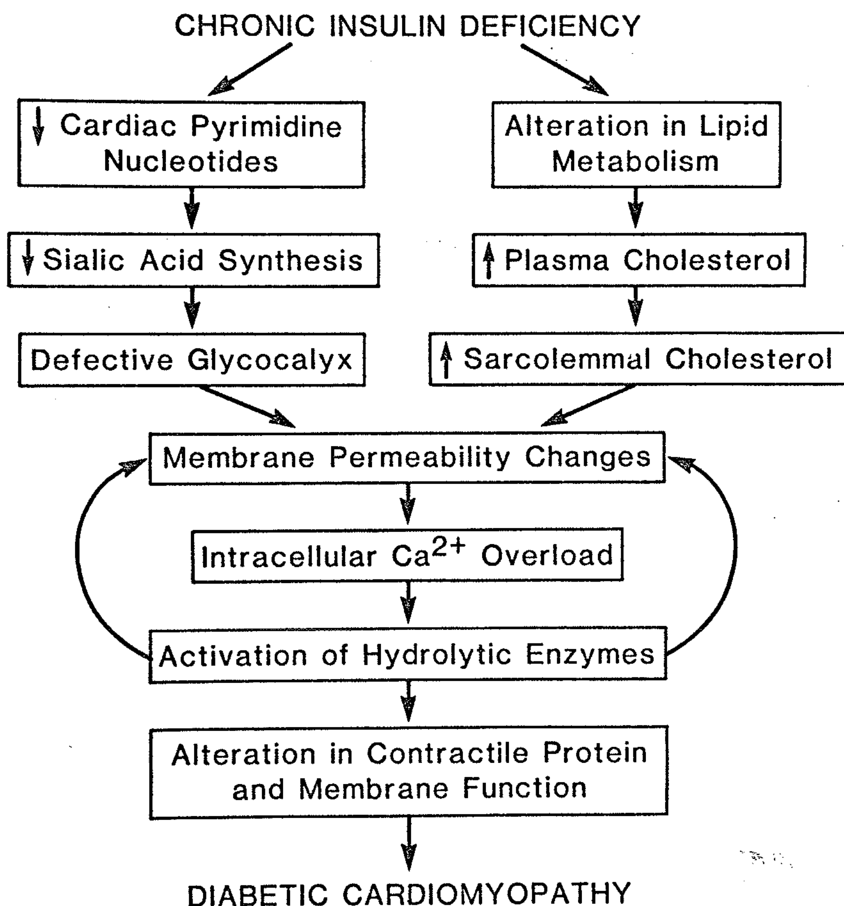

Figure 16. Schematic representation of the events hypothesized to lead to cardiac dysfunction during chronic experimental diabetes. Insulin deficiency over an extended period of time is thought to lead to significant alterations in the sarcolemmal membrane of the heart which could upset ionic homeostasis and result in the features of the diabetic cardiomyopathy. Data from the present study directly support the increase in plasma and sarcolemmal cholesterol, the defective glycocalyx, the alteration in function of various subcellular organelles, and indirectly support the presence of a cell membrane permeability defect. Other aspects of this scheme are supported by observations from other investigators as described in the text.

fatty acids and ketone bodies (Lucas and Foy, 1977; Mansford and Opie, 1968). The abnormally high plasma lipid concentrations consequently result in an increased deposition of fats in the myocardium of diabetic animals (Regan et al, 1974; Opie et al, 1979). The present investigation demonstrated a specific increase in sarcolemmal membrane cholesterol levels. This increase in membrane cholesterol may alter the function of membrane-bound enzymes (Alivisatos et al, 1977) and, more importantly, could threaten cell viability by increasing membrane permeability (Wenzel et al, 1975; Pfeiffer et al, 1978; Peterson et al, 1979).

Insulin has also been shown to have a direct stimulatory effect on the intracellular uracil nucleotide pool (Haugaard et al, 1977). In accord with these findings, chronic lack of insulin has been demonstrated to result in depressed UTP contents in diabetic rat hearts (Gertz and Haugaard, 1979). Since UTP can act as a cofactor in the synthesis of sialic acids (Gertz and Haugaard, 1979), it is possible that this may account for the decreased sarcolemmal sialic acid content observed in diabetic rat heart in the present study. It must be recognized, however, that other factors like increased membrane neuraminidase activity could also be responsible for the depressed sialic acid content. The physiological significance of the decrease in sarcolemmal sialic acid lies in its proposed role in maintaining cell viability and possibly acting as a source of contractile-dependent Ca^{2+} in the heart (Langer, 1978). Although the latter function of the sarcolemmal sialic acid residues is currently controversial (Grupp et al, 1980; Isenberg and Klockner, 1980; Limas, 1977), removal of these carbohydrate moieties from the cell appears to be closely related to increased membrane permeability characteristics (Frank et al, 1977; Langer et al, 1979; Langer et al, 1981; Woods et al, 1982).

It is reasonable, therefore to propose on the basis of the work discussed above, that the decreased sialic acid content and increased cholesterol levels in sarcolemma from diabetic rat hearts may influence membrane permeability and ultimately affect cell viability. Several other lines of indirect evidence may also be thought to support this hypothesis. Firstly, the results of the present study with regard to latent Na^+, K^+-ATPase activity in control and diabetic preparations may represent preliminary evidence for a defect in membrane integrity, as previously discussed. Secondly, a precedent for increased membrane permeability during diabetes has been demonstrated for vascular tissue (Joyner et al, 1981). In addition, an increase in membrane permeability would necessarily result in altered cellular ion distribution. It would be expected that both intracellular Na^+ and Ca^{2+} would increase. Indirect evidence would support the contention that these intracellular ion concentrations are elevated in the heart during diabetes, although no direct determination has been made. Tissue sodium in the heart during diabetes has been shown to be elevated (Regan et al, 1981). Cardiac tissue Ca^{2+} was also reported to be higher during diabetes (Nagase et al, 1981) and , on the basis of the results of the present study, this is unlikely to be bound to the external surface of the myocardial membranes. Electron microscopic evidence of electron dense mitochondrial deposits and contracture zones in contractile proteins (Onishi et al, 1981; Giacomelli and Weiner, 1979; J.H. McNeill, personal communication) suggests intracellular Ca^{2+} may be high. If intracellular Na^+ and Ca^{2+} become elevated, it would be anticipated that subcellular vesicular organelles may swell. Electron microscopic study has revealed sarcoplasmic reticular and mitochondrial swelling (Onishi et al, 1981; Tarach, 1976). On the basis of these arguments, therefore, it is felt that a condition of Ca^{2+} overload may exist in the myocardium

of chronically diabetic animals. This situation would result in a activation of Ca^{2+}-stimulated protease (Dayton et al, 1976) and phospholipase (Pieterson et al, 1974) activities (Dhalla et al, 1982). Protease activities have been shown to be augmented during diabetes (Dahlmann et al, 1979). The alterations in sarcolemmal phospholipid composition observed in the present study suggest phospholipase activity may also be activated. Since many phospholipids are integral to enzymatic function (Panagia et al, 1981), membrane enzyme activity may be significantly compromised. Specific muscle proteases in diabetic rats may be directly responsible for accelerated myofibrillar protein degradation (Dahlmann et al, 1979) which may have produced the electron microscopic evidence of myofibrillolysis (Onishi et al, 1981).

As discussed previously, alteration in membrane vesicular function or contractile protein function would precipitate aspects of the diabetic cardiomyopathy. It is important in this regard to mention that the lesions in function of the various subcellular organelles examined in the present study are not comprehensive and other defects in sarcoplasmic reticular Ca^{2+} transport, for example (Penpargkul et al, 1981; Lopaschuk et al, 1983; Ganguly et al, 1983), may also participate in and contribute to the diabetic cardiomyopathy.

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