SUBCELLULAR ALTERATIONS DURING THE DEVELOPMENT OF HEART DYSFUNCTION IN ALLOXAN-INDUCED DIABETES

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by

Leonard Golfman

Department of Physiology

Faculty of Medicine

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BY

LEONARD GOLFMAN

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

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ABSTRACT

Heart disease remains one of the most serious complications of diabetes mellitus, even with good glycemic control by insulin replacement. Today, heart failure is a leading cause of death in diabetic patients. Several studies have revealed the presence of cardiac dysfunction and ultrastructural abnormalities in chronic diabetes. This type of cardiomyopathy has been associated with varying degrees of subcellular defects which have been suggested to cause the occurrence of intracellular Ca^{2+} overload/impaired Ca^{2+} handling in the myocardium. Although heart dysfunction has been shown to occur in diabetic patients and in experimentally-induced chronic diabetic animals, the significance of subcellular and molecular alterations and their temporal relationship between each other as well as with the observed hemodynamic derangements have not been clearly established. In this study, we examined these issues using rats made diabetic with alloxan (65 mg/kg). Hemodynamic studies revealed that the rate of relaxation (-dP/dt) of diabetic rat hearts was significantly altered at 2 weeks. At nearly the same time, both sarcoplasmic reticulum (SR) and SR ATP-dependent Ca^{2+}-uptake were significantly depressed.

At 4 weeks, diabetic rats exhibited marked reductions in mean arterial pressure, heart rate, +dP/dt and an elevated left ventricular end-diastolic pressure. These results coincided with marked depressions in myofibrillar Ca^{2+}- and Mg^{2+}-stimulated ATPase activities in diabetic rats in comparison with control rats. In addition, by two weeks after alloxan administration, diabetic rat left ventricles possessed a depressed proportion of myosin V_1 and elevated V_3, whereas V_2 remained unchanged over the course of the experiment. Daily

subcutaneous insulin administration (3 U/day) for 4 weeks normalized all the above stated parameters.

Although abnormalities in sarcolemmal (SL) have been identified with respect to Na^+-Ca^{2+} exchange, Na^+-K^+ ATPase and Ca^{2+}-pump activities, the sequence of these changes with respect to each other and SR and hemodynamic derangements have not been clearly elucidated. In this study, both Na^+-K^+ ATPase and SL-Ca^{2+}-stimulated ATPase (Ca^{2+}-pump) were depressed at 10 days following alloxan administration. SL Mg^{2+} ATPase remained unchanged. Na^+-Ca^{2+} exchange (Na^+-dependent Ca^{2+} uptake) along with SL ATP-dependent Ca^{2+} uptake activity were both depressed in alloxan-diabetic rats 14 days after alloxan administration. Insulin administration as outlined previously normalized these above alterations.

The alloxan diabetic rabbit (125 mg/kg body weight) was used as an alternative model 12 weeks after the induction of diabetes. Results showed depressed SR Ca^{2+}-pump and myofibrillar Ca^{2+} ATPase activities in alloxan-diabetic rabbits in comparison to their age-matched controls. It was shown that in diabetic rabbits, only Na^+-Ca^{2+} exchange activity was significantly depressed; SL Ca^{2+}- and Na^+-pump activities did not differ significantly between the control and the diabetic rabbits.

Molecular studies, using various probes for the detection of genes for the alpha-myosin heavy chain (alpha-MHC), SERCA2, $alpha_1$-subunit of the Na^+-K^+ ATPase and Na^+-Ca^{2+} exchanger were undertaken. $Alpha_1$-MHC mRNA was dramatically depressed at 2 and 3 weeks after alloxan administration. However, this was not changed with respect to the controls at 5 and 6 weeks after alloxan administration. In contrast

to alpha-MHC, SERCA2 gene expression was significantly elevated at 3 and 5 weeks after alloxan administration. By 6 weeks, however, there was no significant change in expression of the SERCA2 in diabetic ventricles in comparison to control ventricles. A significant depression at 6 weeks was observed in the expression of the $alpha_1$-Na^+-K^+ ATPase subunit in the diabetic ventricles. No significant changes in the Na^+-Ca^{2+} exchange mRNA expression were observed 2 and 3 weeks after alloxan administration.

The data from this study indicate that alloxan diabetic rats manifest early membrane changes in comparison with controls, and that these changes occur prior to the abnormalities in contractile proteins. There is a strong positive correlation between depressions in the % myosin V_1, systolic hemodynamic indices, depressed myofibrillar ATPase activity, and alpha-MHC gene expression. There is also a strong correlation between depressed diastolic function in diabetes and altered SR Ca^{2+}-uptake. It would appear from these studies that the relationship between sarcolemmal enzyme activities and altered gene expressions for these proteins are not clearly evident; factors such as the phosphorylation status, abundance of high energy phosphates, methylation status, hyperlipidemia, hypoinsulinemia, hyperglycemia and changes in membrane composition (fluidity) play an early, yet undefined significant role.

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I. INTRODUCTION AND STATEMENT OF THE PROBLEM

In recent years, diabetes mellitus in man has been shown to be associated with an increased incidence of heart dysfunction independent of coronary artery disease and atherosclerosis. Preclinical abnormalities in both systolic and diastolic left ventricular function have been detected in a sizeable proportion of diabetics. While the pathogenesis of heart disease in chronic diabetes is uncertain; small vessel disease (microangiopathy) and/or a direct myocardial effect of diabetes have been postulated. Some investigators have described a series of patients with unexplained congestive heart failure who were both hypertensive and diabetic. Their hearts showed myocardial hypertrophy, extensive myocytolic changes with replacement fibrosis as well as interstitial and perivascular fibrosis. Noninvasive studies have shown that high blood pressure may be a factor in the progression of heart disease in diabetic patients. In fact, an increase in mortality due to heart disease was observed upon combining renovascular hypertension and diabetes in rats. It should be pointed out that hemodynamic factors such as microcirculatory hydrostatic pressure in the uncontrolled diabetic state may play a major role in the genesis of microangiopathy and subsequent diabetic cardiomyopathy. Various investigators have reported ultrastructural alterations in hearts from a wide variety of experimental models of diabetes and these have established the occurrence of cardiomyopathy in conditions of poorly controlled diabetes.

Several rat models with streptozotocin-induced diabetes, alloxan-induced diabetes and genetically linked (spontaneously occurring) diabetes are available for studying the pathophysiology of organ

dysfunction, including cardiomyopathy. Although changes in myofibrils and sarcoplasmic reticulum are known in the (predominantly) studies of streptozotocin-induced rat diabetes and alloxan diabetes their temporal relationship is not clear. Abnormalities in the sarcolemma have also been examined with respect to Na^+-K^+ ATPase, Na^+-Ca^{2+} exchange and Ca^{2+}-pump activities; however, the results are scattered and the sequence of these changes has not been well defined. Although various subcellular alterations are known, the molecular mechanisms for such changes remain poorly defined, in both STZ- and alloxan-induced diabetes. Although subellular changes in streptozotocin-induced diabetes are well studied, it is not clear whether these changes are limited to the streptozotocin rat model. Furthermore, although the development of heart dysfunction has been shown to occur in chronic diabetes, the significance of subcellular and molecular alterations in terms of hemodynamic changes have not been clearly established. The studies reported herein were designed to address these issues using alloxan-induced diabetic rats and alloxan-induced diabetic rabbits.

II. REVIEW OF LITERATURE

A. General Consideration of Diabetes and Factors Associated With its Mortality and Morbidity

> "Whether induced by surgical, chemical, endocrine or immunological treatment, ... or whether resulting from genetic manipulation ... models may be extremely informative and helpful but may lend themselves to misuse by equating them to the human disease .... They may be considered glycemia in man but never as a model of diabetes, a disease much more complex than hyperglycemia alone"
>
> -Albert E. Renold

1. Classification of diabetes mellitus

Diabetes mellitus (DM) is one of the leading public health problems in the industrialized world. About one million people in Canada and ten million people in the United States are afflicted with the disease. It is the eighth health-related cause of death. Nearly all the morbidity from diabetes is related to cardiovascular dysfunction - coronary artery disease, hypertension, or renal failure secondary to microvascular disease (1). DM is recognized clinically 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 (2) are classified as insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM).

IDDM is the classification now employed which encompasses the older term of Juvenile-Onset, Ketosis Prone, or Type I to describe diabetic patients. It is characterized by a deficiency of insulin, a well known cause of the hyperglycemic condition, because these diabetic patients

require insulin to maintain a normoglycemic condition. IDDM most often presents itself suddenly early in childhood but it may also occur in adults (3). Polydipsia, polyphagia, polyuria and ketosis are frequent symptoms of the disease and these diabetic patients are normally thin. Several lines of evidence suggest that the IDDM form of diabetes, the destruction of the beta cells of the pancreas which produce insulin, is probably caused by either autoimmune abnormalities or infection (4,5). This may be genetic in origin, but more likely IDDM is predominantly a non-genetic disease (4). On the other hand, 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 also occur in children (4). The patients are usually obese, insulin concentrations are frequently quite normal and therefore insulin therapy is not required (4). The defect in NIDDM is not of the pancreatic origin but is more likely related to cellular resistance to the actions of insulin in the body. This may involve insulin receptor and post-receptor lesions in the cell (6) and is commonly thought to involve some genetic predisposition (4,5).

2. Factors associated with mortality and morbidity in diabetes

From the examination of epidemiological data, it is clear that the diabetic population has a lower life expectancy and higher mortality rates. While the average life expectancy of a diabetic patient has increased dramatically since the introduction of insulin (7), diabetes mellitus still represents a significant health problem in the general population. The factors responsible for the abnormally high mortality are numerous, but much can be learned from statistics accumulated on the

cause of death in diabetic patients. The occurrence of congestive heart failure in the diabetic population has been well documented (8) and dysfunction of the cardiovascular system is considered to be the leading cause of death in diabetics (9). This, however, has not always been the case. Data from the pre-insulin era indicated 20% of deaths in diabetic patients due to cardiac failure and 8% due to coronary vessel disease (10). With the advent of insulin treatment, 51% of the deaths in diabetic patients were due to cardiac failure and 28% due to coronary vessel disease (10). Insulin treatment has virtually eliminated coma as a cause of death in the diabetic population but arteriosclerotic heart disease and other features of myocardial degeneration still remain prevalent. From these statistics, it is evident that death from diabetes is now a clinical problem of cardiovascular dysfunction, with cardiovascular disease accounting for nearly 80% of all deaths in diabetic patients (10,11). It is becoming clear that race does not appear to have an influence upon cardiovascular death rate (12). Both black and white males have a similar increased risk of death from cardiovascular causes if they are diabetic. The presence of diabetes has been reported to increase the change of incurring cardiovascular problems by 2 fold in the male population and 3-5 fold in the female population (13).

a. Myocardial infarction

Diabetic patients suffer from an increased incidence of myocardial infarction (MI) (14-21); the diabetes can increase the possibility of suffering a MI by 2 to 5 times the normal risk (14,16,18,20,21). The increased risk is present in both insulin-dependent diabetes mellitus and noninsulin-dependent diabetes mellitus diabetics (15). In addition, the

sex of the diabetic has an influence on these studies. In nondiabetics, an acute myocardial infarction is more frequent in men than in women (17). However, this protective effect in women is lost with diabetes; myocardial infarction is more frequent in diabetic women than in diabetic men (17). Congestive heart failure and shock are more prevalent during acute myocardial infarction (22) and mortality rate for diabetics after a heart attack is higher than the non-diabetic population also suffering from an acute myocardial infarction (14,15,23-30). After suffering an infarction, approximately 40% of the diabetic patients were reported to die within 1 to 2 months (22,24). A year after the infarction more than half had died (24). In the diabetic population, 5-year survival rates were found to be only 38%. If a subsequent infarction occurred after the first event, the problem was exaggerated. After a second heart attack, over 50% of diabetic patients die within 2 months. Of these patients, 5-year survivorship was only 25% (22). These figures can be compared with 75% survival rate in the non-diabetic population after a first heart attack and 50% after a subsequent event. Overall mortality rates from all causes, including coronary heart disease are higher in diabetic as well as border line diabetic patients (31). This occurs irrespective of the sex of the patient but is more exaggerated in female diabetics or borderline diabetic patients (31).

Various studies have been conducted in order to understand the reasons for the increased incidence of myocardial infarction in diabetics and the poor survival of these patients. A relationship between the glycemic status of these patients and the myocardial infarction has been considered. It has been known for some times that glucose intolerance is frequently associated with myocardial infarction. The occurrence of

myocardial infarction has a distinctly adverse effect on carbohydrate and fat metabolism and often leads to stimulation of the sympathetic nervous system and increased catecholamine concentration (32). Subsequent increases in circulating free fatty acid levels and reduction in glucose tolerance appear to be related to a number of physiological functions - adipose tissue lipolysis, hepatic and muscle glycogenolysis, catecholamine-induced suppression of insulin release, and increased circulating concentrations of growth hormone and cortisol. The net result is that carbohydrate intolerance is common after myocardial infarction, even in nondiabetics. Also, the high concentration of free fatty acid in the acute phases of myocardial infarction may lead to ventricular arrhythmias (33). The suppression of insulin release as a consequence of increased catecholamine activity may decrease glucose utilization by the myocardium which requires this fuel for glycolytic activity (34).

Souton (35) found that previously "normal" patients exhibited carbohydrate intolerance after an infarction. As late as 3 months after an acute infarction, insulin resistance has been demonstrated in patients (36). Datey and Nanda (37) found glucose intolerance in 70% of patients immediately after a heart attack and up to 7-10 days later. Follow-up examinations carried out 1 to 2 years later revealed that the acute myocardial infarction had unmasked latent diabetes in 14% of the patients (37). Thus, it is possible that this poor glycemic status after infarction may further predicate myocardial damage. However, careful control of blood glucose levels immediately after an infarction by i.v. insulin infusion failed to influence subsequent mortality (37). Therefore, it appears unlikely that glycemic status postinfarction

significantly influences myocardial integrity. The problem, however, may lie in conditions prior to and during the infarction event.

In addition to the increased frequency of acute myocardial infarction, there are several factors associated with the infarction itself that appear important in the contribution to the increased mortality of diabetic patients with acute myocardial infarction. First, the size of the infarction is critical for survival. Diabetic patients tend to suffer with a larger infarct compared to nondiabetics (20,21,38). Since infarct size has been closely correlated with cardiac performance and mortality (39), this may then explain the higher mortality rate among diabetics. Several investigators (20,21,38) reported larger infarctions in diabetic women which again correlates well with the increased mortality after a myocardial infarction in diabetic women (20,21,38). However, these observations have been complicated by the findings of Jaffe and co-workers (40) who found that the size of the infarction was smaller in diabetic patients. In spite of this, congestive heart failure was more prevalent in diabetic patients and mortality rate was higher. They concluded that factors other than size of infarct determine cardiac viability and survival in the diabetic population (40). Another factor associated with myocardial infarction which may have more relevance in determining the mortality rate of diabetics is the site of infarction. Maempel (41) found that diabetic patients more frequently had an anteraseptal infarction site than nondiabetics. Weitzman and co-investigators (29) found a higher proportion of anterior site of infarction in diabetics than in nondiabetics. They also reported an increased mortality in patients with an anterior site of infarction (47% mortality) as compared to those suffering an infarction elsewhere (13%

mortality); survival after an anterior myocardial infarction was also shorter.

The insulin status of diabetic patients has also been considered to represent a factor in determining the incidence of myocardial infarction. Sorege et al (42) have found that hyperinsulinism is closely associated with the development of a myocardial infarction. In a more definitive examination, French investigators have concluded that high insulin levels may be an independent risk factor for myocardial infarction and other coronary heart disease complications (43). Smith and co-workers (30) found four significant prognostic factors which may be related to the increased mortality of diabetic patients after a myocardial infarction. These were: (1) pulmonary rates, (2) ten or more ventricular premature complexes per hour prior to discharge, (3) various cardiac symptoms prior to myocardial infarction, and (4) ejection fraction of less than 40%. Thus, diabetes itself constituted a significant prognostic indicator of poor survival after a myocardial infarction.

b. "Silent" heart attack

One of the most important factors suggested to be responsible for the high mortality rate of diabetics who suffer a myocardial infarction is the occurrence of "silent" heart attack. In the diabetic population, a myocardial infarction is frequently painless (22,44-46). Braley and Schonfeld (45) found that an acute infarction can occur in diabetic patients with diminished and less frequent pain or, as was documented in 42% of the diabetics, the pain can be entirely absent. Nesto et al (46) found painless ischemia in a higher percentage of diabetics (72%) compared with nondiabetics (32%) during exercise thallium scintigraphy. Others, however, have found no difference in the incidence of silent

ischemia during exercise after myocardial infarction between diabetics and nondiabetics (47-48). However, Weiner et al (49) felt that when patients with diabetes and coronary artery disease present silent ischemia during exercise, it adversely affects survival. Furthermore, the painless infarction may be particularly relevant in elderly diabetics where the heart attack is frequently associated with atypical symptoms and a poorer prognosis than in the nondiabetic population (50).

It is clear from the above discussion that the implications of painless myocardial infarction are serious. First, the occurrence of an infarction may go entirely unnoticed. Evidence of undiagnosed myocardial infarction in diabetic patients was revealed upon close examination (51). Second, and more importantly, because of the absence of pain, patients fail to recognize that the heart attack has actually occurred and, therefore, these patients are slow in summoning medical help (24). It is well known that the sooner medical attention can be given to an infarcted patients the better are the chances for survival. With the advent of coronary care units, mortality rates after an acute myocardial infarction have dropped significantly because of better and faster medical attention. Prior to the advent of these coronary care units, mortality rates were 38 to 61% (22,27), whereas mortality rates after admittance to coronary care units have been reported to be as low as 24% in diabetic patients (52). An Italian research group (53) has extended these findings on painless myocardial infarction to examine the incidence of transitory myocardial ischemia in diabetes. A significantly grater number of episodes of asymptomatic ST segment changes were recorded in diabetic patients during ambulatory electrocardiogram monitoring than in nondiabetic patients with coronary after disease. This finding is

clinically significant for two reasons. First, like the situation with painless infarction, episodes of asymptomatic ischemia are dangerous because they fail to warn the patient of impending heart trouble. Furthermore, ischemic heart disease in the absence of angina pectoris is difficult to treat when chest pain is one of the most common ways to judge the efficacy of treatment (53).

The lack of pain during myocardial infarction in diabetics is thought to be due to a cardiac autonomic neuropathy dysfunction which exists in many diabetic patients (54-56); this causes an increased anginal threshold, presumably as a consequence of autonomic and sensory neuropathies (57). It is well known that sympathetic efferents mediate v pain, whereas the relation between pain and ischemia and parasympathetic activity is less clarified (58). Sympathetic gangliectomy of the upper three to four ganglia on both sides of the thoracic sympathetic chain resulted in relief of anginal pain in 75% of patients (59,60). However, in some patients, angina pain persisted after sympathectomy, which raised the possibility of the presence of cardiac vaga efferent fibres. The question that arises, then, whether a complete separation into sympathetic and parasympathetic involvement is feasible (61). Tests for isolated sympathetic afferent function are lacking. Quantitative techniques used in diabetics with vagal dysfunction may unmask the abnormal sympathetic function (62). It can be assumed that autonomic dysfunction, which is significant in diabetics with painless myocardial infarction, is probably related to the parasympathetic pathway (8).

c. Congestive heart failure

Diabetic men were found to have twice the incidence of congestive heart failure as nondiabetic men; diabetic women were at five times as

great a risk as nondiabetic women (63). In addition, diabetic patients suffer from an increased incidence of congestive heart failure after a myocardial and they are also more likely to incur cardiogenic shock complications after the infarction (22,64-67). Even when patients with prior coronary or rheumatic heart disease were excluded, diabetic subjects had a 4 to 5-fold increased risk of congestive heart failure. Furthermore, this risk persisted after age, blood pressure, weight, and cholesterol values, as well as coronary heart disease, were taken into account (13,63,66). On the basis of these findings it appeared that the excessive risk of heart failure in diabetic patients is caused by factors other than accelerated atherogenesis and coronary heart disease. It has been suggested that a diabetic-induced cardiomyopathy is involved.

d. Coronary thrombosis and stroke

Coronary thrombosis with or without infarction has been reported to occur more often in the diabetic population than in the general population. It has been shown that women are particularly at risk. Diabetic women are 14 times as likely to suffer from coronary thrombosis than nondiabetic women (68). Thrombosis was also found to be a significant contributory factor in recovery of the diabetic patient from a myocardial infarction (68).

Stroke and cerebrovascular incidents are more frequent in the diabetic population than in the control population as evidenced in epidemiologic and post-mortem studies (11,69-73). Diabetic women were three times as likely to suffer a stroke as nondiabetic women (73); the severity of stoke was greater in diabetics as well (72,73). The incidence of stroke which resulted in permanent neurological damage was twice as high in diabetics as in nondiabetics even when atherosclerotic

vascular disease was taken into account (73). Diabetic patients were found to have a greater number of stroke-related deaths (72). Epidemiological studies demonstrate that diabetes mellitus carries an overall 2 - 6-fold increased risk of thrombotic (but non hemorrhagic) stroke. Diabetes is believed to cause 7% of deaths due to stroke, and cerebrovascular disease may be present in 25% of patients dying with diabetes (74). Diabetes increases risk of atherosclerosis in all vascular beds including the brain. Hypertension, a major risk factor for stroke, is more common in those with diabetes (74). However, the increased risk of stroke in diabetic individuals persists even when corrected for other concomitant risk factors, i.e., hypertension, that occur more commonly with diabetes mellitus (75).

e. Hypertension

The incidence of hypertension has also been reported to be higher in the diabetic population (18). This study also found that the incidence of myocardial infarction in the diabetic hypertensive population was higher than that exhibited by the nondiabetic hypertensive group (18). Factor et al (76) reported that the association of diabetes with hypertension may substantially increase myocellular damage and interstitial fibrosis. They described dense interstitial connective tissue myocytolysis and scarring in the myocardium of nine hypertensive diabetic patients with congestive heart failure and minimal obstructive coronary artery disease.

The prevalence of hypertension in diabetics has been reported in various ethnic groups (77,78). Frequently, hypertension coexisting with diabetes is a significant risk factor. The prevalence of hypertension in diabetes ranges from 40 to 80% (79). Hypertension is a

serious risk factor, accelerating peripheral vascular disease, stroke, nephropathy, retinopathy and cardiac disease (79). Since hypertension is known to be an important risk factor in heart failure (80) and, as stated above, diabetes is also an independent risk factor even in the presence of hypertensive complications, these compounded problems may further increase the chance of cardiovascular abnormalities.

In long standing diabetes mellitus, nephropathy is an important cause of hypertension, mostly in type I. On the other hand, Hasslacher (81) has shown that hypertension accelerates the course of diabetic nephropathy. Hypertension accelerates (82) the evolution of nephropathy following proteinuria which, in turn, is preceded by the onset of microalbuminuria (83).

In an epidemiological study involving thousands of Polish diabetic patients living in Warsaw (84) a familial prevalence for diabetes, coronary heart disease, and hypertension was investigated. No increase in the incidence of coronary heart disease and hypertension was found in close relatives of insulin-dependent diabetics in comparison to the general population. However, in relatives of the obese or nonobese insulin-dependent diabetics, significant increases in the prevalence of these lesions were demonstrated. Further observations argued against environmental factors playing a significant role and suggested that hypertension and diabetes may have a genetic origin and the type of diabetes mellitus may influence the coappearance of these diseases. It is important to point out that the prevalence of hypertension in the diabetic population is a controversial issue. Although several studies have found that hypertension was more common in the diabetic population than in the general population (18,76,79,85,86), others have found no

difference (87). This disagreement, however, does not lessen the significance of the previously discussed findings that hypertension compounds the cardiovascular risk factor which is already abnormally high in the diabetic patient. Thus is appears that there are numerous factors which may be responsible for the alarming statistics of cardiovascular problems associated with diabetic patients. In the final analysis, however, three major factors appear to largely account for the increased incidence of cardiovascular dysfunction during diabetes. These factors, namely major vessel diabetes in the form of atherosclerosis, microvascular alterations, and a primary myopathic disorder are thought to be the major conspirators implicated in varying degrees, in concert or acting independently in both human and experimental diabetes.

B. Animal Models of Diabetes

1. Use of animal models in diabetes research

It now has been over 100 years since Minkowski and von Mering in 1889 first demonstrated that diabetes could be induced by pancreatectomy using a dog as an experimental model. Work on diabetes has been continued using animals with the hallmark of successful extraction of insulin from the pancreas of a dog by Banting and Best in 1921, showing the hypoglycemic effectiveness. Other animal species, predominantly rodents, have been used since then to test the action of insulin and other hypoglycemic agents and to induce diabetes by destroying the pancreatic B-cells with cytotoxins. It is important at this time to explain what should be meant by the phrase "animal model of diabetes". This is a term often used when reporting work with diabetic animals and extrapolating its significance to human diabetes. The term "model" is not a reproduction of human diabetes in an animal neither

does it represent the full range of aberrations or complications observed in human diabetics (88). In this sense, a rodent with diabetes may disappoint some clinicians studying diabetes who expect a scaled down replica of the human syndrome with all its abnormalities. The potential of an animal lies in the opportunity to explore specific morphologic, biochemical, immunologic, or metabolic parameters which are not accessible in the human by biopsy or autopsy. Therapeutic measures such as drugs and transplantation, may be applied prior to their use in humans (88). It should be stated that the conclusions should thus be confined to the pathogenesis of a particular derangement or its correction, because the full equivalence of the whole disease is rare.

Most of the medical research focused on diabetes mellitus is conducted by employing various types of animal models of this disease. As discussed by Grant et al (89), there are two fundamental factors that support the justification for the continued use of animals in diabetes research. First, a large proportion of the research on diabetes today requires intensive tissue analyses or the manipulation of external variables which would render experimentation on diabetic human patients absolutely infeasible on ethical grounds. This point is best exemplified by the problems involved in research dealing with the diabetic cardiomyopathy. The amount of information which one may obtain from the in vivo analysis of cardiac function in human diabetics is limited. One may ascertain that the heart is functioning subnormally but it is impossible to gain any significant insight into the reason for the dysfunction. Cardiac tissue must be removed from the body in order to understand many crucial aspects of muscle mechanics, function of organelles at a subcellular level, membrane composition, and metabolic

status. A major portion of the heart must be removed in order to provide enough tissue to analyze these factors. Obviously, such research is impractical in human diabetic patients. Yet, this information is essential if the investigator is to gain some insight into the mechanisms responsible for producing the cardiac dysfunction. It is only when a mechanism is established to explain the dysfunction that effective treatment can be developed and initiated. Without the availability of a substantial amount of tissue for conducting intensive analysis, in most circumstances, a researcher can only hope blindly for mechanisms and treatments of the disease.

Second, the information one can obtain from studies on diabetes in animals is usually far more valuable than that obtained from the study of human diabetics. From a pathological viewpoint, the use of animals in diabetic research offers several critical advantages. The appearance of spontaneous, genetically determined diabetes in specific animal strains can be studied over several generations of animals under highly controlled conditions. In man, this work is fraught with difficulties, if not totally impossible. An animal model of diabetes also affords the researcher with the opportunity to study the disease during the lifetime of the animal - prior to the overt appearance of the diabetic state, and during the entire course of the disease. The study of diabetes in man, is, for the most part, an acute examination which consequently yields limited data. To the researcher, having access to a diabetic model described above is invaluable in learning more about why the disease becomes clinically apparent, the characteristics of its development, and what kind of prophylactic measures may be taken during the prediabetic period to prevent the appearance of the disease. Thus

an animal model of diabetes remains as the only useful model of the disease if solutions to the etiology of diabetes and its complications are to be found. It should be emphasized that animal experimentation is conducted because it is the only viable option available with which to obtain information on the characteristics, management and eventual prevention of the diabetic condition.

Various experimental models which may closely approximate the clinical situation have been developed. Generally, the animal models of diabetes can be subdivided into two broad categories: (i) spontaneous, genetically determined diabetes, and (ii) experimentally-induced diabetes. While each type has advantages and disadvantages regarding its use and applicability to the clinical picture, one must reiterate that unfortunately, no model of animal diabetes precisely replicates all the features of the human diabetic condition (90); thus, any data obtained from animal studies of diabetes must be interpreted with caution. The major focus of this section will deal primarily with the second broad category of animal models of diabetes, namely, the experimentally-induced diabetic animals. For a comprehensive review of spontaneously occurring diabetic animal models (which may be genetic and/or immunologic in origin), the reader is referred to several excellent reviews (88-106).

Although clinical studies give us some indication of the factors that may cause heart disease in diabetics, a better understanding can only be obtained from well-controlled experimental studies. Experimentally-induced diabetes in many animal species has been more frequently employed in the field of diabetic research. 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 (90,107). Injection of specific viruses has also been demonstrated to elicit beta cell necrosis and a diabetic condition in mice (108-110). However, two drugs, alloxan and streptozotocin (STZ), have gained widespread use as diabetogenic agents. Because of their general acceptance in the field of diabetes research, a discussion of their usage, with a particular attention focused on alloxan will now follow.

Alloxan and STZ are chemicals selectively toxic to the pancreatic B-cells. Alloxan is a pyrimidine with structural similarity to uric acid and glucose, while STZ is deemed as glucose with a highly reactive nitrosurea side chain. The B-cells toxicity of alloxan was discovered by accident while testing the nephrotoxicity of uric acid derivatives in rats and rabbits (111-112). Dunn et al (111) first reported the diabetogenic action of alloxan. These researchers were attempting to explain the mechanisms that accounted for kidney dysfunction. A model of renal failure was needed and they examined agents which, upon injection, could cause such a lesion. Since it was known at that time that uric acid elicited toxic effects on the kidney, they were investigating the effects of uric acid and some related derivatives on renal integrity. Injection of the uric acid derivative, alloxan, into rabbits did produce lesions in the kidney. However, many animals died soon after treatment from causes which were obviously distinct from renal complications. Further investigation (111) revealed a derangement in plasma glucose

homeostasis and necrotic damage in the pancreatic B cells. Homologues and compounds related to alloxan are also capable of causing diabetes. These include N-methyl propylalloxan, ethyl propylalloxan, alloxantin, dimethylalloxantin, diethylalloxantin, dialuric acids, and methyldialuric acids (112,113).

Alloxan (2,4,5,6-tetraoxohexahydropyrimidine) has a complex chemical structure and it exists in several tautomeric forms. Alloxan in the monohydrate form is easily dissolved in water and slightly acidic. In water and, at the neutral pH or alkaline pH, this chemical is fairly unstable and can be converted to alloxanic acid (114) unless stored below freezing. A more practical and convenient method of storage is simply to buffer the solution with citrate buffer (pH 3 - 4.5). This will increase the stability of alloxan in solution so that it can be stored conveniently for long periods of time between 0 and 5°C. This is not only convenient but it also appears to increase the success rate of producing diabetes in rats (115). Some researchers, however, have reported variable results using older, stored solutions of the drug (116); most investigators now do not store either drug but prepare fresh solutions and use them immediately. Delivery of the drug in a buffered citrate vehicle appears to be important to reduce nonspecific, toxic side effects of the drug and significantly increases the success rate of producing diabetes in rats (116). The half-life of STZ in serum is 15 min (90) whereas the half-life of alloxan is much sorter; it was estimated to be less than 2 minutes in the body whereas in vitro measurements found it to be 0.9 minutes (114). Due to the relatively short half-lives of the drug, the route of entry of the drug into the body of the animal is limited. Injections (intramuscularly,

subcutaneously or intraperitoneum) of these drugs are not recommended since these drugs may be significantly degraded within the body before they ever reach the target organ of their action - the pancreas. This perhaps explains the observation that mice given intraperitoneally dosages of STZ, which would have been twice the lethal dosage if given intravenously, had very little toxic effect (118). The method of choice is intravenous administration of alloxan and STZ. The tail vein of an animal like the rat is a fast, convenient injection site used by many investigators (119-121) but the femoral vein (122,123), intracardiac (124), and other sites of injection have been employed without difficulties.

Pincus et al (125) have reported that the rate of delivery of alloxan can affect the development of diabetes. For each dosage of alloxan tested, the mortality rate and the number of rabbits which exhibited diabetic symptoms decreased as the rate of injection into the vein was slowed. The metabolic status of the animal at the time of injection can also influence the potency of the drug (126,127). Animals which are fasted 24 hr prior to the delivery of the drug are more sensitive to the its effects. In practical terms, this means that the injection of a determined amount of alloxan will result in higher resting blood glucose levels in fasting animals than in non-fasting animals. This response is probably due to the protective effect of blood glucose against the actions of the drugs.

The concentration of the drug delivered and the frequency of the injection of the drug also influences the degrees of hyperglycemia subsequently exhibited by the animal. A single injection of a moderate concentration of the drug is recommended over multiple injections of an

equal amount of drug because animals are reported to develop a certain amount of resistance to repeated injections of lower doses (128). The amount of drug delivered is closely related to the severity of the hyperglycemia experience by the animal. Junod and co-workers (129) reported that STZ produced diabetogenic effects at dosages between 25 to 100 mg/kg body weight of the animal. An extensive analysis of the blood metabolites of animals after varying doses of STZ was carried out by Schein et al (117). They observed that as the dosage of STZ given to the animal increases, a fall in serum insulin levels was accompanied by a rise in blood glucose and lipids. Interestingly, plasma triglycerides showed a dramatic increase after 100 mg of STZ/kg of body weight, whereas plasma-free fatty acids exhibited a striking rise in concentration after a dosage of 75 mg STZ/kg. Total ketone bodies were unaffected by an injection of 50 mg/kg; this is an important point if the researcher wishes to employ a non-ketotic diabetic model. Other investigators have reported that ketonuria was observed in animals only at STZ doses of 100 mg/kg body weight (129). Generally, as the dose of alloxan or STZ given to the animal increases, the derangement in blood metabolites also increases. An exception to this lies in carbohydrate metabolites in the blood. Even after dosages of up to 150 mg of STZ/kg of body weight of the rat, blood lactate and pyruvate concentrations were relatively unaltered (117). Doses of alloxan or STZ in the amount of 100 mg/kg and higher are usually lethal in rodents. Unless the diabetes is effectively controlled by exogenous insulin treatment, severely ketotic animals, after receiving 150 mg of STZ/kg body weight will not survive more than 3 days post injection.

The changes in blood metabolites which occur as a function of the

amount of diabetogenic chemical administered are accompanied by severe abnormalities in pancreatic integrity as well. A low dose of alloxan/STZ of 20 mg/kg body weight affected pancreatic insulin concentrations only negligibly (130). However, at a dosage of 30 mg/kg body weight, pancreatic insulin levels were reduced by about 50% (130). Increasing dosages of the drug resulted in a linear decrease in pancreatic insulin at 65 mg of drug/kg body weight, whereupon only about 10% of the pancreatic insulin remained (130). Pancreatic insulin concentration is an important determinant of glycemic homeostasis in the blood. Abnormal glucose tolerance can be observed when pancreatic insulin levels are depleted by 30% but fasting hyperglycemia and glucosuria are evident only when pancreatic insulin levels are reduced by about 70% (129). Arison and colleagues (131) have demonstrated that the size of the dosage of diabetogenic chemical may influence its specificity as well. In rats given 65 mg of drug/kg of body weight, light and electron microscopic examination revealed that B-cell degranulation was the only observed alteration in pancreatic structure. However, in pancreas samples taken from animals injected with 100 mg of drug/kg of body weight, nonspecific lesions were observed throughout the specimens. These lesions may have represented alpha-cell inactivation in addition to the B-cell necrotic sites. Thus, it was concluded that larger dose of alloxan or STZ may reduce the specificity of the drugs producing B-cell necrosis.

In order to learn something about how the drugs produce their effects, studies of agents which may inhibit the diabetogenic actions of these drugs have been undertaken. Shein et al (118) were the first to document the protective effect of nicotinamide against the diabetogenic action of STZ. Related compounds like nicotinic acid, N-methyl

nicotinamide, nicotimuric acid, and glutathione did not block the effects of STZ. The ability of nicotinamide to block the diabetogenic action of STZ was found to related to the concentration of both drugs administered and dependent upon the length of time which elapsed between the injection of STZ and the injection of the nicotinamide (129). Nicotinamide delivered up to 30 min after the injection of STZ still did not block its diabetogenic action, although less potently than if given earlier. In contrast, nicotinamide was ineffective in blocking of the diabetogenic effects of alloxan if it was given 10 min after injection of alloxan (132). Other agents which have been sown to block the diabetogenic action of alloxan and STZ are: D-glucose, D-mannose, 3-O-methyl-D-glucose, D-fructose, D-galactose, alpha-methyl-D-glucoside, D,L-glyceraldehyde, D-xylose and 2-deoxy-D-glucose (118,132,133). 3-O-methyl-D-glucose has obtained the most widespread use as blocker of the actions of STZ in studies of heart dysfunction during diabetes (134,135). As was the case with nicotinamide, in order to be optimally effective, the 3-O-methyl-D-glucose must be delivered to the animal immediately prior to the injection of STZ (127).

2. Acute physiological changes after the injection of alloxan and STZ

The immediate effect of an injection of alloxan or STZ on blood metabolites and hormones is extremely varied depending upon the length of time which has elapsed after the injection. Administration of alloxan/STZ is most effective in different species at doses ranging from 50 - 200 mg/kg in rats, dogs, mice (88,136-139), Chinese hamsters (140), monkeys (141-142), miniature pigs (143) and rabbits (136,137). STZ has been found to be more effective than alloxan in certain species, such as Guinea pigs (144-145) and Syrian hamsters (146), which do not develop

permanent hyperglycemia after alloxan. On the other hand, alloxan has been found to be effective in inducing diabetic in rabbits which are resistant to the diabetogenic effects of STZ (147). Animals treated with STZ/alloxan, although highly insulin deficient, do not usually require insulin treatment for survival. In fact, a mild diabetic state may be induced in rats by a single low dose of STZ, for example of approximately 35 mg/kg (88). Alloxan and STZ produce irreversible functional B-cell damage within minutes and structural changes within hours in most rodents, dogs, cats, rabbits, monkeys, sheep, cattle, fish and birds (88). Up to 1 hr after injection, the animal suffers a transient hypoglycemic period (148) followed an hour later by a severely hyperglycemic episode (117,132,138,149,150). The reasons for these initial fluctuations is unclear since plasma insulin levels appear to be normal (132); liver glycogenolysis has been suggested as a probable cause (138). However, 6 to 7 hr after the injection the pancreas release a massive amount of insulin which causes a dangerously hypoglycemic period in the animal (117,132,149,150); if severe enough, this hypoglycemic episode can kill the animal. Dosages of alloxan/STZ in the range of 70 mg/kg body weight or less will rarely elicit a life threatening hypoglycemic reaction at this stage and due to the severity of this hypoglycemic period, alloxan or STZ should not be given to fasted animals. If the animal survives this period, it will exhibit elevated blood glucose levels by 12 to 48 hours after the injection.

The cause of hyperglycemia after alloxan or STZ injection is specific necrosis of the B-cells of the pancreas. Alterations in the B-cell morphology closely parallel the disturbances in glycemic homeostasis immediately subsequent to the injection. B-cell mitochondria show

evidence of disruption 10 min after the injection of alloxan (151). Within 45 min after the injection, freeze-fracture study of the B-cell plasma membrane reveal a depletion in the intramembranous particles (152). As early as 1 hr after STZ administration, B-cell damage has been observed (149). By 7 hr post-injection, when insulin release is large and blood glucose concentration is low, B-cell necrosis becomes evident (149). This observation has led to the formulation of a hypothesis that insulin is not actively released during this period but instead leaks from a severely damaged cell (132). If the cell cannot maintain its membrane integrity, it also cannot regulate the release of the stored insulin. This would account, therefore, for the massive increase in plasma insulin concentration and would explain the observation of Veleminsky and colleagues (130) that pancreatic insulin content was less than 1% of control values 24 hr after the initial injection of the diabetogenic agent. However, not only the extent of the derangement in plasma glucose after the injection was found to be a function of the amount of alloxan or STZ given to the animal, the extent of pancreatic damage was also be closely correlated with glycemic status. Abnormal glucose tolerance was observed when pancreatic insulin was depleted by about 30% (129). More severe indications of an imbalance in glucose homeostasis (e.g., as indicated by fasting hyperglycemia or glycosuria) were only observed when pancreatic insulin was depleted by approximately 70%. Thus, these considerations established pancreas as the site of action of alloxan and STZ for producing the diabetogenic effect.

Up until the last six years, the mechanism by which alloxan or STZ elicited their specific necrotic action on the pancreatic B-cells had largely been unknown; however, several plausible theories had been

advanced. Like and Rossini (128) presented evidence from a study in which they employed multiple subdiabetogenic doses of STZ and examined islet ultrastructure. Their results suggest that the diabetogenic action of STZ may involve a cell-mediated autoimmune response, which correlated well with Notkin's (108) theory of a viral-induced human diabetic state. Boquist (151) proposed a hypothesis to explain alloxan diabetogenicity that involved a complex interaction with B-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 mitochondria. This would result in an impairment of oxidative phosphorylation function and ultimately in cellular necrosis. In 1988, Okamoto and collaborators (153) put forward a concept for a uniform mechanism of action of alloxan, STZ and similar molecules. They considered fragmentation of B-cell DNA as the crucial event, caused by accumulation of superoxide and OH radicals and/or DNA alkylation. Breaks in the DNA strands were considered to be responsible for the deterioration in insulin synthesis and secretion. This also starts immediately the repair processes, involving the activation of poly (ADP-ribose) synthase and the associated NAD utilization. Okamoto et al (153) maintain that the NAD depletion is so precipitous that if becomes irreversible and results in virtual cessation of NAD-dependent energy and protein metabolism and thus cell necrosis. This edifying concept is strongly supported by the preventive effects of nicotinamide supplementation and free radical removal by various scavengers (such as superoxide dismutase, dimethylthiourea and citiolone (154-156)), which inhibit the activity of poly ADP-ribose synthase.

3. Chronic metabolic and physiologic changes in alloxan- and STZ-diabetic animals

Early after the injection of alloxan/STZ, a condition of chronic hyperglycemia is permanently established. Associated with this condition is a generalized disruption of metabolic and hormonal homeostasis. The most obvious feature to be observed is a drastic loss in body weight in the diabetic animal. Body weight can be reduced by up to 40% from control animals (157-159) but food consumption in the diabetic animals is increased by as much as twofold (159,160). Fluid intake increases in the diabetic animals to 500% of that observed in control animals (159,160). Hough and co-workers (160) found urine output increased over tenfold in STZ-induced diabetic rats. The weight of stool samples from diabetic rats was doubled in comparison to control animals.

Increased hepatic glucose production and gluconeogenic enzyme activity are prominent and reflect the virtual absence of insulin (161). The derangement in blood metabolites is severe in uncontrolled, chemically induced diabetes. These changes are not restricted merely to a large elevation in blood glucose due to reduced glucose utilization are a consequence of insulin deficiency, circulating lipids and proteins are also affected significantly. The extent of these effects are dependent on the dosage of alloxan or STZ administered to the animal. High circulating glucose levels in the blood essentially represent the only major alteration in carbohydrates observed in chemically induced diabetic animals; even relatively high drug dosages do not produce significant changes in blood lactate or pyruvate (117,162). Low doses of alloxan or STZ given to the animal do not change plasma free fatty acids (FFA), cholesterol, triglycerides, ketone bodies, or phospholipids too

drastically (89). However, larger doses of such drugs will result in an elevation of plasma triglycerides, cholesterol, and phospholipids carried by the very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions in diabetic animals (89,117,163-166) due to the hepatic conversion of the mobilized FFA to triglycerides, as well as to the delayed peripheral disposal of VLDL and chylomicrons (164,167). This is related both to the decrease in insulin-dependent lipoprotein lipase activity and to alterations in the apoprotein content of lipoproteins (167,168), particularly in apo E and C, necessary for the recognition and efficient lipolysis of these particles at the sites of their uptake. It should be emphasized that the HDL fraction is increased in insulinopenic animals, contrary to its lowering in diabetic humans. This fraction is the main carrier of circulating cholesterol in many animal species.

With respect to lipid metabolism, alloxan and STZ-diabetic rats mobilize stored triglycerides, as evident from the rise in the intracellular lipase activity in adipose tissue which is followed by a pronounced outflow of FFA and rigorous FFA oxidation in the muscle, liver, and kidney. The activity of carnitine acyl CoA-transferase, regulating the transport of FFA-CoA esters into the mitochondria, is elevated in rat liver and kidney (169,170). This is related to insulin deficiency since it does not occur in hyperinsulinemic obese rats, which are characterized by elevated FFA flow to the liver. Ketosis and ketanuria are present, more often in alloxan- than STZ-treated animals, but they do not usually progress to lethal ketoacidosis. This is a significant distinction from the autoimmune, insulin-dependent BB rats and NOD mice and indicates that a residual insulin secretion persists in alloxan- and STZ-treated rats. In fact, some regeneration of B-cells

including B-cell adenomas and other neoplasms (171,172), which prevents a totally unrestrained FFA and ketosis, has been documented. This "spontaneous" amelioration of diabetes limits the usefulness of STZ- or alloxan-treated animals to three to six months after the induction of diabetes and requires scrutiny of pancreatic function in long-term experiments.

Alloxan- and STZ diabetic animals manifest a negative nitrogen balance related to enhanced proteolysis in muscle and other tissues, coupled with lower protein synthesis (173) and growth failure related to decreased somatomedin activity (174). The increase in proteolysis is caused by a rapid mechanism (173) and by a slow but long-lasting activation of a myofibrillar protease (175), which is only gradually abolished by insulin (176). Increased protein catabolism with inflow of amino acids to the liver feeds the pathway of gluconeogenesis and accelerates ureagenesis. Total protein and albumin concentrations in plasma from chemically induced diabetic animals appear to be relatively normal (177-179). However, urea levels in the blood due to ureagenesis are significantly higher in diabetic animals (121). Serum amino acid levels are substantially changed; valine, leucine, isoleucine, and methionine concentrations are increased in serum from diabetic rats that were administered a moderate dosage of alloxan (124,180,181). Conversely, alanine, glutamic acid, serine, threonine, glycine, tryptophan, lysine, and arginine levels were decreased in blood samples from diabetic animals (124,180,181).

Untreated, chemically induced diabetic animals have also been reported to be in a condition of moderate hyperphosphatemia, have normal plasma magnesium, and arterial pH (160,182). In contrast, one

investigation (183) has found elevated plasma-magnesium levels in experimental animals as the duration of the diabetic condition was increased. Early in the diabetic condition (i.e., 2 weeks post-injection) rats exist in a negative calcium balance. However, 6 weeks after the induction of the diabetic condition, the animals compensate by increasing their intestinal absorption of calcium (160). This produces a condition of hypercalcemia in the diabetic rats (160). In addition, some trace metal concentrations are also altered during diabetes (95,184). It should be stated that in addition to alterations in plasma insulin levels, other hormones in the plasma are also altered in chemically induced diabetic animals. Plasma glucagon, somatostatin, corticosterone and norepinephrine levels increase significantly (160,179,182,185-194). In contrast, thyroid hormone, parathyroid hormone, vitamin D, growth hormone, progesterone, and luteinizing hormone concentrations are all lower in plasma samples from diabetic animals (123,178,182,195-201). Because these hormones are important for the regulation of a number of physiological processes in the body, this hormonal imbalance is considered to affect the metabolic growth, fertility, and many other bodily functions of the animal.

4. Limitations to the use of STZ and alloxan

Several limited considerations must be kept in mind when employing the drug-induced diabetic animal model. For example, the susceptibility of animals to STZ is age and sex dependent. Mordes and Rossini (202) found young rats resistant to all but massive doses of STZ, and as the age of the animal increased lower STZ doses were needed to elicit a diabetic condition. However, this may be species dependent since Riley et al (203) found precisely the opposite circumstances in mice injected

with STZ. Species-dependent differences in the resistance to alloxan administration (204) and toxic dosages of STZ (205) have been reported. Rabbits are resistant to the diabetogenic effects of STZ but not alloxan. Guinea pigs have been previously thought to be resistant to diabetogenic agents but data (206) have demonstrated that guinea pigs developed significant and prolonged glycosuria and became polydipsic after STZ injection; insulin levels in the plasma were significantly depressed. These features strongly suggested that these animals were diabetic; however, serum glucose levels were normal.

Several other precautions may be taken when using the chemically induced diabetic animal model. For example, long-term investigations of STZ- and alloxan-induced diabetes in rats must be undertaken with the knowledge that these animals may revert spontaneously to a euglycemic state (88,207) in 3 to 6 and even up to 18 months after drug injection. Although these observations have been contested (208), it is wise to test periodically the glycemic status of diabetic animals when studied over an extended time period. In addition, the diet of the animal prior to injection can also influence its susceptibility to drug-induced diabetes (126,127).

Furthermore, the specificity of the diabetogenic chemicals for the B-cells of the pancreas remains a crucial issue. Alloxan was isotopically labelled with ^{14}C and its distribution throughout the bodies of mice was monitored after injection (209). The radioactivity was found in tissue samples from the renal cortex, arterial walls, bone, tendons, and the pleuralas as well as the peritoneal cavities. These data suggest that although alloxan may preferentially accumulate in the pancreas, it is far from specific and may have the potential to have

nonspecific toxic effects. Several other investigators have reported that alloxan is capable of direct toxic action in the kidney and lung which have no relation to the diabetic state of the animal (112,210). These nonspecific effects may not apply to STZ. Junod and colleagues (149) found that the B-cytotoxic effects of STZ and alloxan were similar but the action of the former appeared more specific. Arison et al (131) reported that STZ administration produced more reproducible pancreatic lesions and the general toxicity was lower than when alloxan was given to the animals. However, at higher dosages, both agents began to lose their B-cell specificity and pancreatic alpha-cell inactivation was suggested.

It seems appropriate at this time to discuss some of the important differences between the two drugs, namely alloxan and STZ. Although both drugs elicit a diabetic state by a direct toxic action on the pancreatic B cells, certain characteristics of the two drugs are different. Firstly, it is important to recognize that the mechanism of the toxic action of each drug on the B cell may be different (89,211). This is emphasized by the difference in the number and type of compounds which can block the diabetogenic action of either drug. Furthermore, the toxic effect of alloxan on the B-cell may be slower than that of STZ (130). Alloxan has direct effects on mitochondrial function, whereas STZ does not elicit such an action. The metabolic state of the animal after injection may also be different depending on the drug administered. Hofteizer and Carpenter (213) found that alloxan administration produced consistently higher blood glucose values than STZ given the same molar dosages. Perhaps the most important difference between alloxan and STZ is that ketosis and ketonuria are present more frequently in alloxan - than in STZ-treated animals (89,117,214). These effects, ketosis and

ketonuria do not, usually, however, progress to lethal ketoacidosis. Plasma lipid profiles and cardiac content of various metabolites can also be very different in the two models of drug-induced diabetes (130,214).

Mansford and Opie (214) observed that the metabolic patterns in the circulating blood and heart tissue of rats with diabetes induced by STZ or by alloxan were strikingly different. In comparison to STZ diabetic rats, plasma-FFA and blood ketones were approximately 3- and 15-fold higher in alloxan diabetic rats. In addition, heart glycogen content was double in alloxan versus STZ diabetic rats. In addition, they found elevated contents of glucose-6-phosphate, fructose-6-phosphate and citrate and lowered levels of fructose-1,6-diphosphate in alloxan versus control rats. In STZ rats, only citrate and glycogen levels were lower with respect to control hearts and the other glycolytic metabolites did not differ compared to control. The abnormal patterns of glycolytic intermediates and the increased contents of citrate and glycogen in hearts from acute alloxan-diabetic animals are thought to result from the high circulating FFA and ketone levels. The normal pattern of glycolytic intermediates in STZ diabetes and the failure of cardiac glycogen and citrate levels to increase are thus in keeping with the absence of ketosis in this type of diabetes mellitus. Since many patients with diabetes mellitus are hyperglycemic but not ketotic, it might be suggested that the patterns of tissue metabolism in such patients may more closely resemble those found in STZ diabetes rather than alloxan diabetes.

Overall, one must assess the pros and cons regarding the use of drug-induced diabetic animals. The advantages to using drug-induced model of diabetes in comparison to other models of diabetes are:

convenience, less cost, high reproducibility, ability to control the severity of hyperglycemia, as well as ability to use a wide variety of animal species. The disadvantages are as follows: it is not a "natural diabetes", nonspecific toxicity of the drugs, can only study an insulin-deficient model of diabetes, cannot study genetic transference of the disease as well as generally impossible to study the "pre-diabetic" stage of the disease (89). In spite of these both the STZ and alloxan model of diabetes are widely used; both drugs are readily attainable and the speed with which diabetes is produced after administration of either drug allows him/her great flexibility in choosing a starting time for an investigation.

C. Ultrastructural and Microvascular Changes in Diabetic Cardiomyopathy

"Primary cardiomyopathy" has become a loosely used term in clinical circles to describe cardiac dysfunction under a wide variety of circumstances. Literally, primary cardiomyopathy is a disease of the myocardium which is present independent of external factors. It occurs not because of insufficient coronary perfusion of the myocardium associated with occlusive coronary atherosclerosis, but is expressed in the form of hemodynamic and contractile abnormalities and structural derangement. In many instances, the etiology of the cardiomyopathy will be unknown (215). It is emphasized that numerous clinical and epidermiological reports have confirmed that human diabetics appear particularly susceptible to heart failure, which is the leading cause of death in these patients. Factors that appear largely to account for cardiac problems and increased incidence of cardiovascular dysfunction in chronic diabetes include atherosclerosis of the coronary arteries, microangiopathy and autonomic neuropathy. However, it has also become

apparent that these factors are not always responsible for the cardiac problems associated with diabetes. For example, a significant number of diabetic patients who do not develop atherosclerosis, microangiopathy, and autonomic neuropathy still suffer from cardiomegaly, left ventricular dysfunction, and clinically overt congestive heart failure (216). These results suggest that a specific cardiac muscle disease may also occur during diabetes and this disease (diabetic cardiomyopathy) is likely a consequence of a direct effect of insulin deficiency on the myocardial cell function.

The diagnosis of diabetic cardiomyopathy is considered in those patients in whom coronary artery disease, alcoholism, hypertensive cardiovascular disease or other etiological factors (such as valvular heart disease) producing myocardial dysfunction has been rule out. Evidence to support an impairment of performance due to a lesion of cardiac muscle itself (not due to vascular pathology) comes from post-mortem findings, abnormalities in contractile function in absence of major vessel disease and ultrastructural derangement of cardiac tissue. Accordingly, it is intended to examine the involvement of myocardial ultrastructural changes, microvascular changes, impairment of contractile function (animal and human, invasive and non-invasive studies) and subcellular defects which are manifested in the diabetic cardiomyopathy.

1. Myocardial ultrastructural changes in diabetes

A variety of different types of cells having unique and specialized functions which are responsible for the structure and performance of the heart. Cardiomyocytes, smooth muscle, endothelial, fibroblasts, and other nonmuscle cells represent just a few of the various cell types found in the heart. The cardiac muscle cells are the primary functional

component of the myocardium and determine the force generation of the heart. It is for these reasons the cardiomyocytes have attracted the most attention in studies on diabetic cardiomyopathy. Although these contractile cells are not predominant in the heart in number, cardiomyocytes compose some 80 to 85% of the volume of the heart (217,218) and account for more than half of the its weight (219).

Various techniques are available to examine the structure of the myocardium; these include morphological methods, freeze-fracture cytochemical methods, and vadioiodination techniques (220-224). One of the most informative methods employs electron microscope for examining the structure of the myocardial cell (220-224). The use of electron microscope has allowed investigators to examine the myocyte's organelles in extreme detail; the principle organelles which have been targeted in various studies of diabetic animals include the contractile proteins, mitochondria, sarcoplasmic reticulum (SR), the transverse tubular system, sarcolemma and the nucleus. Since each organelle, by virtue of its own, unique function, contributes to the maintenance of the myocardial cell in a functional and reliable state, a lesion in any of these subcellular organelles would result in a compromised condition and, thus ultimately in a functional defect. The use of electron microscopic and morphometric techniques has revealed that the myofibrils and the mitochondria account for nearly 50%, and 33 - 3% of the rat's total myocytes cell volume (253), respectively, with much smaller volumes occupied by other subcellular structures (transverse tubular system - 1%, SR - 2%, nucleus - 5%, sarcolemma (very low), lysosomes (very low) (219,225)).

A number of studies have observed derangement in the contractile

proteins in the hearts of diabetic animals. These alterations have been described as loss of contractile proteins (225-228), degeneration of myofibrils (229,230,234), lysis of myofibrils (230-232), derangements in the Z-band region (230,233,234), disorganization of myofibrils (226,227,230), and contraction of sarcomeres (227-229,234). These findings have been reported in spontaneous diabetes (225,230,231) and in chemically-induced (Alloxan/STZ) diabetes in rats and rabbits (226-229,231-234). The implications of these ultrastructural alterations are that cardiac performance in diabetes may be depressed severely by an inefficiency or inability of the myofibrillar proteins to generate contractile force development.

The membranous systems in the heart also appear to be abnormal during diabetes when examined under the electron microscope. The SR system shows signs of disruption in the spontaneously diabetic KK mouse heart (230). In drug-induced diabetes in animals, many investigators have reported swollen SR tubules (228,231,232,235), mild edema immediately adjacent to the SR (236) and loss of SR (226,227). In addition, the membrane which envelopes the cell is also disturbed by the diabetic condition. It was observed in one report that portions of the sarcolemmal membrane had lifted away from the myocardial cell surface; there was also considerable blebling and vacuolization immediately adjacent to that area (237). Since similar changes have been noticed in experimental models of cardiac dysfunction due to intracellular Ca^{2+} overload (238), it is possible that the observed alterations in diabetic heart may be a consequence of the occurrence of intracellular Ca^{2+} overload. These sarcolemmal changes may be involved in increased permeability characteristics of the heart observed during diabetes

(239). In addition, local thickening of the cardiac sarcolemma during diabetes has also been reported (225,232). In studies with alloxan diabetic rats (226,227) complete loss of transverse tubules have also been reported. Furthermore, cell to cell contact has been observed by a number of investigators to be altered in the hearts of diabetic animals. In drug-induced diabetic animals (226-229,232,240) the intercalated disk region is visibly separated and widened, along the fasciae adherents (226,227), with maintenance of both desmosomes and gap junctions (226,227,232). This may disturb intercellular communication and may partially explain, a least from an ultrastructural standpoint, the enhanced arrhythmogenic capacity of the diabetic heart (241).

Mitochondrial abnormalities are the most common ultrastructural disturbance reported in the heart during diabetes. Mitochondrial swelling is frequently observed (229,230,232-236,240,242); however, one study observed mitochondrial shrinkage (225) and Thompson (227) reported that mitochondrial size and architecture were rarely altered in alloxan-induced diabetic rats. On the other hand, generalized mitochondrial disruption (225,228) is evident in the form of vacuolization of the mitochondria (243), clearing of the matrix (229,243), separated cristae (232,234), and lysis of both inner and outer mitochondrial membranes (243). This damage could be the result of increased lysosomal activity in heats from diabetic animals. An incorporation of lysosomal membranes into the mitochondrial matrix has been observed (229). An increase in the number of electron-dense particles in the mitochondria has also been reported (225,236); this may reflect an accumulation of Ca^{2+} ions. Other structural alterations have also become evident from electron-microscopic examination of the diabetic animal heart. Lysosomes have

been shown to increase in number n myocardium from diabetic animals (228,229,243). The lysosomes were reported to be found more frequently near large amounts of glycogen and lipid droplets in the cell (243). Alterations in the nuclei of myocardial cells during diabetes have also been noticed. Condensation of nuclear chromatin (229) and the presence in the nuclei of perichromatin granules with a bright "halo" (232) have been described. Seager and co-workers (229) reported a folding of the nuclear membranes. An abnormal increase in perivascular space around the nucleus has also been observed (228). Giacomelli and Wiener (225) have reported degenerative nerve endings in the diabetic heart.

2. Changes in the storage of metabolic substrates in the myocardial cell

Evidence from electron-microscopic studies indicate that there are changes occurring with respect to the storage of metabolic substrates in the myocardial cell from diabetic animals; these changes corroborate similar findings by traditional biochemical methods. Several studies have observed an increased number of lipid droplets in myocardial cells from diabetic animals (225-229,232,234-236). These lipid droplets have been noticed to be situated in close proximity to the Z-line (228,244) in both chemically-induced and spontaneous models of diabetes. Another common observation in the diabetic myocardium is an increase in the electron-dense glycogen particles (228,232,243,245). Again, this fining using the electron microscope correlates well with biochemical evidence of an increase in myocardial glycogen content during diabetes (246).

The extracellular space in the heart contains a matrix of polyanionic, protein-mucopolysaccharide sites (247); collagen is the primary protein in the extracellular matrix. Collagen fibrils may run

in very close apposition to the membrane surface in smooth muscle cells and myocardial cells and, in some cases, may even become embedded in the membrane wall (247). Collagen is an important extracellular structure and is thought to maintain myocardial function by a number of mechanisms. Due to its viscoelastic properties, collagen fibrils may transmit and distribute force generation during muscular contraction (248). Collagen may also influence muscle function through an interaction with Ca^{2+} ions as it may serve as an extracellular Ca^{2+} binding site (247) or alter the diffusion of extracellular Ca^{2+} into muscle cells (249). In disease states like experimental hypertension and hypertrophy, increases in collagen synthesis by smooth muscle cells have been demonstrated (250). However, it should be pointed out that there still exists some controversy regarding the status of collagen in hearts of diabetic animals. Where some investigators report evidence in favour of an increase in collagen content of the heart in diabetes (136,227,231,233,251,252), others have found no change (230,245). It has been suggested that the nature of the diabetic condition could perhaps represent an important factor n the accumulation of collagen in the heart during diabetes (8). Particularly, the moderate and chronic models of diabetes appear to exhibit a consistently higher myocardial collagen content (233,251,252). It is the contention of many investigators that an increase in collagen content inhibits myocardial distensibility and alter cardiac pump performance.

3. Factors influencing the development of ultrastructural changes in the heart during diabetes

Although it is becoming clear that insulin-dependent diabetes produces a progressive pattern of ultrastructural changes in the

myocardium of different diabetic animal models, it should be stated that ultrastructural derangement in the myocardium does not always accompany diabetes mellitus. Reports of diabetic effects on myocyte ultrastructure, for example range from no effect (252) to myocytolysis and contraction bands (228). In this regard, several factors have been identified in the diabetic state which may affect the development of ultrastructural abnormalities in the heart. These include the duration and severity of diabetes, the type of diabetes, and the presence of other accompanying diseases in the animal. Modification of any of the above factors will result in dramatic changes in the expression of structural damage to the heart during diabetes mellitus.

One of the most closely examined factors which influences the progression of ultrastructural damage in the heart is the length of time that the animal is exposed to diabetes. In short-term diabetes, ultrastructural changes in the heart are minimal. Electron-microscopic examination of sections of the myocardium in rats 2 to 4 days after the induction of a diabetic state by alloxan injection revealed no alterations in the structure of cellular organelles (244). Only an increase in the lipid droplets was observed so soon after alloxan injection (245,224). In the drug-induced diabetic rat model, it is difficult to ascertain precisely when significant ultrastructural derangement can be documented. As early as 7 days after the induction of diabetes, mitochondrial swelling and dilation of SR tubules have been reported (235); however, others have reported no changes in myocardial ultrastructure as late as 6 weeks after injection of STZ (228). Tarach (232) however, reported increases in cardiac lipid and glycogen deposition as well as swelling of the fascia adherents of the

intercalated disk in 6-week alloxan-diabetic rats. Thompson (227) observed loss of myofibrils, disorganization of remaining myofibrils, disruption of banding pattern, loss of SR elements and transverse tubules, and separation at the intercalated along the fascia adherents in 6-week alloxan-diabetic rats. Some investigators have found gross ultrastructural alterations at 8 weeks (229), 12 weeks, and 24 weeks (227,228) after the induction of the diabetic state with either STZ or alloxan. Thus, although the duration of the diabetes does influence myocardial ultrastructure, the presence of abnormalities represent a gradual and very subtle progression. The changes may be so subtle that conventional electron-microscopic techniques may not provide adequate resolution to detect them. This view is supported by the observation that functional depression of the heart preceded the manifestation of ultrastructural abnormalities in the heart (228).

In the spontaneously diabetic mouse (C57BL/KsJ dbt/dbt) Giacomelli and Weiner (225) documented the ultrastructural alterations of the heart as a function of the age of the animal. As early as 5 weeks of age, myocardial cells exhibit mitochondrial changes, disruption of sarcomeres and focal thickening of the sarcolemma; severe damage was present in the hearts of 6 to 7 month-old diabetic mice. In contrast, little evidence of damage to the arterial vessels and capillaries was present at 5 weeks of age in the diabetic animals (225). At 12 weeks of age, some alterations were observed in endothelial and smooth muscle cells; these included dense mitochondria, focal areas of lysis, and increased amounts of endoplasmic reticulum and golgi bodies. By 24 and 28 weeks of age, the diabetic mouse heart exhibited an increase in collagen content, destruction of capillary lumen, mitochondrial loss, and thickened basal

lamina of arteries (225). The most significant finding was the difference in the time-course of the appearance of ultrastructural defects in the myocardial cell as opposed to alterations in the arterial vessels and capillaries. The observation that changes in myocardial cells preceded those in the vasculature (225,227,229) represents important evidence that diabetes is attended by a primary cardiomyopathic condition.

It should be pointed out that not every investigator has observed ultrastructural derangement in hearts from diabetic animals (251-253). Part of the reason for this discrepancy may involved the severity of the diabetic state. One investigative group (251,252) has consistently used a alloxan-induced model of diabetes in dogs which does not exhibit resting hyperglycemia but does show abnormal glucose tolerance. They have found little or no evidence of changes in the ultrastructure of cellular organelles in the hearts of these animals. In addition, no changes in the vasculature were noted. Alterations in myocardial composition (an increase in collagen content and lipid droplets) were the only unusual feature associated with this type of diabetes. The severity of the diabetes may represent a plausible explanation for the presence or absence of ultrastructural changes in the heart during diabetes (8). This importance of severity of insulin-dependent diabetes was examined in a time-course study of alloxan-diabetic rats (226). Even with the same degree of hyperglycemia, glycosuria, polydipsia, and polyuria, moderately diabetic animals did not develop the degenerative ultrastructural changes seen in myocardium from more severely diabetic rats. These include decreased cardiocyte size, loss and disorganization of myofibrils, and loss of sarcoplasmic reticulum and transverse tubules.

The hypothesis that the severity of diabetes may represent a plausible explanation for the presence or absence of ultrastructural changes in the heart during diabetes was given further indirect support by studies which have examined myocardial ultrastructure by electron microscopy in insulin-treated diabetic animals. Daily insulin administration to diabetic animals reversed all myocardial ultrastructural defects, which were associated with short-term diabetes (235). This was also true for the most part in diabetes of longer duration. However, some mitochondrial abnormalities and vascular resistance to a normalization in ultrastructure in response to insulin treatment were observed (236). These studies stress the increased difficulty in normalizing vascular ultrastructural alterations with insulin as opposed to changes in cardiac muscle cells.

The presence of other diseases in diabetic animals or patients has also been shown to augment cardiac ultrastructural damage. The hypertensive-diabetic animal is one such example. A far greater incidence and severity of ultrastructural abnormalities were observed in diabetic animals which were also hypertensive (253). Disorganization of sarcomeres, increased numbers of mitochondria, and alterations in the intercalated disc region in the myocardial cell from hypertensive-diabetic rats were reported (253). A thickened pericapillary basal lamina and an increase in endothelial pinocytotic vesicles in myocardial capillaries were also found in these animals. All of these changes were less severe in the hypertensive rat or in the diabetic rat in comparison to those observed in the hypertensive-diabetic rat hearts (253). Similar findings were reported using the light microscope on myocardial sections obtained from hypertensive-diabetic rats (254) and diabetic patients

with hypertension (76). Considering the reported prevalence of hypertension in the diabetic population (7), the presence of cardiac ultrastructural alterations in the diabetic community may be more widespread than originally assumed.

From the foregoing discussion it is evident that ultrastructural changes in the heart are dependent upon the duration, severity, and type of diabetes present. Alterations in myocardial ultrastructure can occur in the absence of arterial or capillary changes whereas increase in the number of lipid droplets appears to be one of the earliest abnormalities detected in the diabetic cardiac muscle cell. Sarcomere disorganization, myofibrillar loss, and mitochondrial swelling and clearing are among the more common ultrastructural lesions reported. Less frequently observed but still notable are nuclear abnormalities and T-tubule and SR swelling. Sarcolemmal alterations and separation of the intercalated disc region have also been reported. Vascular defects, when observed, usually appear in the form of thickened arterial or capillary basal lamina. Micropinocytotic activity associated with the capillary wall has also been reported. These lesions are aggravated by the presence of hypertension in the diabetic subject. A relationship of these ultrastructural abnormalities in the heart during diabetes to cardiac functional depression has been challenged by others; however, the presence of such damage does provide clear, undeniable evidence that the heart is undergoing a process of pathological regression.

4. Contribution of small vessel disease in diabetic cardiomyopathy

The cardiocyte, while functionally the most important element, its function, and subsequently the function of the heart is critically dependent upon proper support by the other components of the heart. In

particular, the cardiocyte is dependent upon the ability of the microvasculature to supply metabolic substrates and hormonal factors, to remove metabolic wastes, and to regulate both the ionic and osmotic environment of the tissue. Abnormalities in the capillaries and arterioles take the form of local constrictions of the vessel, abnormal vascular flow patterns, aneurysms, vessel permeability changes, and alterations in reactivity to various stimuli. These injuries may lead to reperfusion damage in the myocardium and have been implicated by many as a primary cause of cardiac contractile dysfunction and failure. The possibility that this complication may be a contributing factor in heart dysfunction during diabetes has received much attention and have led to much controversy.

Several studies have reported no atherosclerotic plaque formation in the myocardial arteries of diabetic animals (229,230,255). In addition, no structural changes in smooth muscle or endothelial cells in the small arteries, arterioles, or capillaries of the heart during diabetes (8 wk, STZ-diabetic rats) were evident (229). Furthermore, no basement membrane thickening has been observed in hearts from diabetic KK mice (230). However, these above results are in conflict with several other animals studies which document a thickening of small arteries and the basal lamina of capillaries in the heart during diabetes (227,231,232,245,256). A thickened densa in the presence of a loss of the lamina lucida has been observed in myocardial capillary walls during diabetes (256). Pinocytotic activity in the capillary walls also been reported (227,233,256), with increased number and size of micropinocytotic vesicles (227,256). The explanation for the controversy in findings in the vasculature of the heart during diabetes in

experimental animals may be related to the duration and severity of diabetes. It is also of interest that insulin administration to chronically STZ-diabetic rats did not prevent capillary basement membrane thickening in the myocardium (256). On the other hand, insulin administration to chronically alloxan-diabetic rats (227) reversed the diabetic-induced capillary abnormalities. The endothelium was smooth and normal thickness, and the micropinocytotic vesicles were of normal size and distribution (227).

In the past, the role of small vessel in the pathogenesis of diabetic heart in humans has been questioned by some investigators. Resnecov (257) considered the increased incidence of heart failure among diabetic patients probably to be caused by microangiopathy. The rarity of small vessel disease in non-diabetic patients with cardiomyopathy was also stressed. Regan et al (259) examined 10 uncomplicated diabetics and found significant luminal narrowing of extramural or intramural coronary arteries in one patient. All had accumulation of PAS (periodic acid-Schiff) material in the left ventricular interstitium, interstitial fibrosis and hypertrophy. Factor et al (261) noted microaneurysms of myocardial capillaries. Thickening of the basement membrane (basal lamina) was reported by Silver et al (262) in postmortem samples and by Fischer et al (263) in tissues removed during bypass surgery. Shirley et al (258) included 12 patients with diabetes mellitus in their group of 139 patients with primary myocardial disease. Based on biopsy findings, autopsies were performed in two diabetic patients and there was no small vessel involvement. Ledet (260) reported no significant changes in capillaries of postmortem human myocardium of young juvenile diabetics. Negative results in reporting small vessel disease may be due to

technical difficulties or to the imperfection of the technique. Biopsy techniques may miss focal changes of the myocardium (79). For example, in the studies by Yoneraich and Silverman (264), four paraffin blocks from seven areas were taken; each block was sectioned at 6 um and four sections were stained. They found small vessel disease alone in 50% of the 36 diabetics.

The causes and importance of capillary basement thickening as a result of diabetes have been dealt with extensively elsewhere (265,266). It has been suggested that this thickening occurs independently of the lowered insulin levels and is perhaps due to hyperglycemia and/or other metabolic and hormonal imbalances. This might explain why insulin treatment failed to prevent changes in STZ-diabetic rats as reported by McGrath and McNeill (256), however, it doesn't explain the contrary findings by Thompson (227) with alloxan-diabetic rats. Many investigators have suggested that the increased microvascular permeability to macromolecules, such as plasma proteins, plays an important role in the pathogenesis of diabetic complications in several organs (267). There are numerous reports in the literature on increased microvascular permeability to macromolecules in diabetic patients and experimental diabetic animals. While the mechanism remains to be elucidated, it has been suggested (256) that the observed increase in the number of micropinocytotic vesicles could help explain the increased vascular permeability reported in diabetes and may thus influence the cardiac dysfunction.

Most of the evidence reported to support an involvement of the microvasculature in the depression of cardiac performance during diabetes is still, unfortunately, largely conjecture. Many past studies have

reported abnormalities in heart function, or EKG recordings from diabetic patients and, after observing no evidence of coronary stenosis or ischemic heart disease, have concluded the cause of this dysfunction to be microvascular in origin (63,274-276). However, no direct evidence supports such a conclusion in these studies.

The existence of focal changes in microvessels are not sufficient to account for the diffuse myocardial degeneration with interstitial fibrosis, which is a pathomorphological feature of diabetic cardiomyopathy (268). Furthermore, the presence of a functional microangiopathy (increased microvascular permeability) in the diabetic myocardium has not been identified until the last year in 1993 when Yamaji et al (268) provided evidence for increased capillary permeability in the diabetic rat myocardium. They used an in situ perfusion method, which is the most direct way of investigating microvascular permeability to macromolecules in rat myocardium in the diabetic state. They reported that capillary permeability to albumin was markedly increased in the diabetic rat myocardium because of enhanced vesicular transport. They hypothesized that this may play an important role in the pathogenesis of diabetic cardiomyopathy. The increased capillary permeability in diabetic rat myocardium shown in their study, was a remarkable functional changes seen in almost all capillaries. This functional change in capillaries, observed diffusely in diabetic myocardium, is hypothesized to be pathogenetically much more significant than focal structural abnormalities of microvessels.

It has been suggested that abnormally increased extravasation of plasma constituents such as albumin may cause myocardial edema (increased interstitial matrix), which would be followed by interstitial fibrosis

during a long period of disease (269). Both interstitial edema and fibrosis, which are the most histopathological features of diabetic myocardium (227,270-272) may contribute to the development of diastolic dysfunction through increased ventricle stiffness. Several investigators have suggested the significance of advanced glycosylated end product in the pathogenesis of vascular complications (273). Advanced glycosylation end products accumulated on vessel-wall proteins and collagen may decrease protein removal, increase new protein deposition, and stimulate cellular proliferation in the interstitium by forming crosslinks with extravascular plasma proteins (273). Such studies have provided evidence that one of the principal pathophysiological features of diabetic complications is the morphological and functional alterations of microvessels, called "diabetic microangiopathy". Morphological microangiopathy of both the relatively large intramyocardial vessels as well as arterioles and capillaries has been identified in diabetic myocardium of experimental animals and human diabetic patients. However, the exact relationship of microangiopathy to the pathogenesis of diabetic cardiomyopathy remains as yet, controversial. It has been recently suggested that functional microangiopathy, in association with the formation of advanced glycosylation end products, may play an important role in the pathogenesis of diabetic cardiomyopathy in a manner similar to that for retinopathy or nephropathy.

D. Heart Function During Diabetes

As a pump, the heart of a diabetic patient is in a compromised condition. Several techniques have been employed to evaluate cardiac function in both humans and experimental diabetic animals. Noninvasive methods, including electrocardiography, phonocardiography, graphic

recording of carotid pulsation, echocardiography, and radionuclide perfusion have been used individually or in some cases, in combination, in order to measure the performance of the heart. These methods are carried out in a clinical setting with little compromise or discomfort to the patient; however, interpretation of the data is difficult and conclusions from such studies are often limited. The use of invasive techniques extends the quantity and quality of information which must be obtained regarding force generation by the cardiac muscle. Cardiac catheterization, heart-lung preparations, isolated hearts, ventricular wall strips, interventricular septal preparations, and isolated papillary muscles represent only a few of the methods and models whereby cardiac contractile force generation may be directly monitored in an invasive manner. Because of their invasive nature, ethical considerations restrict their use to animal studies. These data, unfortunately, also have limited value, in this case with respect to the direct application of these animal results to heart function in humans. Nonetheless, in the past two decades, a number of both invasive and noninvasive studies have been conducted in human diabetic and experimental animals in order to more clearly define the alterations of cardiac physiology and to evaluate pump performance in diabetes and diabetic cardiomyopathy. In this section, it is planned to deal with different aspects of cardiac performance including (i) human diabetic studies, (ii) animal studies, and (iii) methods to normalize cardiac function in diabetes.

1. Human diabetic studies

Results from fundamental analyses of cardiac performance during diabetes have been reported by various investigators. Resting heart rate was similar in control and diabetic subjects (277,278) or higher in the

diabetics (279,280). Manipulation of external variables unmasked significant differences in heart rate. For example, the ingestion of small quantities of alcohol did not alter heart rate in control subjects but stimulated the heart rate in diabetics (277). Ambulatory monitoring of heart function for a 24-hr period revealed higher diurnal heart rates in diabetic men (281). This was especially true for those patients suspected of autonomic neuropathy. Under intense exercise stress, Abenavoli and co-workers (282) found that diabetic subjects without evidence of clinical heart disease had lower maximal heart rates than control subjects. Blood pressure measurements were shown to be dependent upon the presence of atherosclerotic complications in the arterial system during diabetes. Hypertension in the diabetic population has been reported (18). In relatively asymptomatic diabetic men, systolic blood pressure tended to be higher in those patients with autonomic neuropathy but lower in diabetics considered to have peripheral neuropathy (281). Diastolic blood pressure changes may correlate with pathological alterations. Shapiro and colleagues (283) discovered that the posterior wall of the heart was thicker in diabetic patients if the diastolic blood pressure was between 100 to 125 mmHg than if it was less than 100 mmHg. Cardiac hypertrophy has been considered as an indicator of clinical heart disease and part of the pathology of diabetic cardiomyopathy (284,285).

The majority of investigations on cardiac function in diabetic patients have used systolic time intervals as a measure of cardiac performance. The measurements are accomplished by simultaneous recording of the electrocardiogram, phonocardiogram, and carotid pulse tracing. The critical parameters within the systolic time interval are the pre-ejection period (PEP), left ventricular ejection time (LVET), the

PEP/LVET ratio, the conduction time, and the isovolumic contraction time or isovolumic relaxation time. Cardiac dysfunction has been associated with increases in PEP, PEP/LVET ratio, and isovolumic contraction time whereas stimulated cardiac performance was associated with decrease in these systolic time interval measurements (286,287). However, it is important to note that systolic time intervals have a limited value in individuals in which the carotid pulse wave was difficult to record, or when septal defects or vascular disease were present (286). The application of systolic time interval measurements to cardiac performance in diabetes has revealed significant defects in almost all the values. Systolic time intervals (288), especially the ratio of PEP/LVET has been shown to correlate with invasively determined parameters of contractile performance such as ejection fraction (287). Several independent investigators have reported an increase in PEP (277,283,286,289,290), an increase in the PEP/LVET ratio (242,277,283,286,289-293), a prolonged isovolumic contraction time (286), and increase in conduction time (286), and a prolonged isovolumic relaxation time (283,291). This was observed in both insulin-dependent diabetics and non-insulin dependent diabetic patients; however, one study noted more pronounced defects in the former (283). Although one group found no correlation between the duration of diabetes and changes in systolic time interval (286), another study found that in diabetic patients under 20 years old there was a significant correlation between these indices (291). Alterations in systolic time intervals in diabetic patients have also been correlated with glycosylated hemoglobin levels (289) and blood glucose levels (293). Uusitupa and co-workers (294) found that when blood glucose concentrations were better controlled, PEP and PEP/LVET ratio declined

and LVET increased towards control values. Upon closer examination of their data, they found that only those diabetic patients whose fasting blood glucose concentrations had improved $\geq$ 3 mmol/l demonstrated significant changes in systolic time interval. Thus, these studies would support a close relationship between cardiac dysfunction, as evidenced by abnormal systolic time intervals, and the diabetic condition (especially hyperglycemia).

Doppler echocardiography has been increasingly utilized to assess systolic and diastolic function of the left ventricle in a variety of conditions (295). Pulsed Doppler ultrasound interrogation of mitral inflow velocities affords a simple and reproducible method for evaluating diastolic and systolic variables that correlate with radionuclide and invasive techniques (296-298). Doppler echocardiography is independent of factors that alter ventricular geometry and can be easily employed. Ventricular diameters are calculated from the echocardiogram recordings during systole and diastole in order to determine the ejection fraction which is an indicator of cardiac pump fraction. A larger than normal ejection fraction would represent an augmentation in contractile function of the heart whereas a decrease in the myocardial ejection fraction has been considered to be an indicator of heart disease. Zoneraich and colleagues (290) could find no indication of any defects in left ventricular function in a group of diabetics using echocardiography even though systolic time intervals demonstrated significant dysfunction. Only in a selected subgroup of diabetic patients did significant left ventricular dysfunction become evident by echocardiography (290). Notwithstanding these problems, several investigations have revealed defects in cardiac pump performance in diabetes using echocardiography.

The percent of myocardial fractional shortening was lower in diabetics than non-diabetics (282,292,299). The ejection fraction was also subnormal in diabetic patients (292,299). The end-systolic left ventricular volume was greater in diabetics (299). The left ventricular wall movement and mitral valve opening had less of a temporal relationship than usual (275). In fact, mitral valve opening was often delayed in diabetic subjects and, in some patients, the outward left ventricular wall movement was recorded prior to the opening of the mitral valve (275). The latter finding is consistent with ischemic heart disease, and in view of the patency of large vessels, it may be interpreted as indicating small vessel disease (275); other observations support this conclusion (292). However, echocardiograms from diabetic patients in other studies were suggestive of left ventricular hypertrophy (300) and altered compliance, possibly due to an accumulation of interstitial glycoproteins (300,301).

Radionuclide angiography has also been employed to asses cardiac performance in control and diabetic patients. The majority of investigations using radionuclide angiography have observed no difference in the resting left ventricular ejection fraction between control and diabetic patients (301-304). However, manipulation of several external variables revealed significant defects in cardiac performance in diabetic patients. If blood glucose levels rose, the mean ejection fraction at rest rose significantly higher than in the normoglycemic period (301). The explanation of this effect is unknown. If diabetic patients were exercised, significant depressions in the ejection fraction were observed in comparison to control patients (302,303). This was observed frequently enough in one study (302) to allow investigators to conclude

that as many as one third of their diabetic patients exhibited subclinical left ventricular dysfunction. Another investigation unmasked cardiac dysfunction in diabetics only when stress-tested by cold stimulation (305). The mechanism responsible for this effect was unclear, although autonomic complications may represent one possible factor (305). Abenavoli and colleagues (282) observed a decrease in percentage of myocardial fractional shortening in diabetic patients. In all of the studies, macrovascular disease was eliminated as a factor involved in the cardiac dysfunction.

The presence of a defect in left ventricular contractile function during diabetes is supported by other investigators who have used other measurement techniques including catheterization and contrast ventriculography. Myocardial dysfunction during diabetes is evidenced by a decrease in ejection fraction (306,307), and increase in left ventricular end-diastolic pressure (242,270,306,307), an increase in end-diastolic volume (242,270,307), a decrease in cardiac index (307), mean systolic ejection rate (307) and stroke volume (242,270), and an increase in the left ventricular end-diastolic pressure to volume ratio (270). Increasing the afterload of the heart in diabetic resulted in higher filling pressures without altering the stroke volume (270). Large vessel complications were not considered to contribute to these indices of cardiac dysfunction in these studies. Instead, small vessel disease (306,307) and alterations in ventricular compliance due to the accumulation of collagen (242,270) have been suggested to account for the depressed cardiac performance during diabetes.

In the preceding discussion, impairment of the left ventricular systolic and diastolic functions have been detected frequently with type

I diabetes and has been related to specific diabetic complications and the duration of disease. Overall, few reports on early changes in left ventricular function in cardiac-asymptomatic diabetic patients exist. Some of them have stated that diastolic function is more commonly impaired than systolic function and that diastolic dysfunction may be the primary abnormality in patients with diabetes (295,308). In the very recent study of Raev (278) it was found that myocardial damage in cardiac asymptomatic type I diabetic patients with diabetes affects diastolic function before systolic function. In order to minimize the influence of factors other than diabetes, on left ventricular function, this study excluded heart failure and/or arterial hypertension, coronary artery disease, other cardiac and noncardiac diseases, above average physical activity, and the use of drugs with the exception of regular doses of insulin. A high prevalence of diastolic dysfunction (impaired active and passive properties of left ventriculum) with preserved systolic function in young cardiac-asymptomatic type I diabetic patients was observed. The results further showed that diastolic dysfunction was twice as common as systolic dysfunction (27% vs 12%). Of the diabetic patients with systolic dysfunction, 83% had impaired diastolic function, whereas only 30% of diabetic patients with diastolic dysfunction had systolic damage. On the other hand, only 3 of 157 diabetic patients (1.9%) had systolic dysfunction with preserved diastolic function. Furthermore, diastolic dysfunction, represented by the interval from minimal left ventricular dimension to mitral valve opening, was seen in diabetic patients approximately 8 years after onset of diabetes and systolic dysfunction represented by fractional shortening, after about 18 years. In addition, diastolic dysfunction, represented by isovolumic-relaxation time, was

found in the presence of mild complications and systolic dysfunction, represented by fractional shortening, was only found in the presence of more severe complications.

In many other disorders, such as congestive heart failure (309), hypertension (310) and coronary artery disease (311), systolic abnormalities have been preceded by diastolic dysfunction. Diastolic dysfunction with intact systolic function in patients with diabetes has been reported by many authors previously (283,312-315). Thus, these findings also confirm those of Raev and co-workers (278) that the abnormalities of diastolic function may therefore be an earlier sign of diabetic heart muscle disease than impaired systolic function. In terms of clinical implications, the results of Raev's study (278) indicate that the cardiopathic process in patients with diabetes affects diastolic function before systolic and show that an isolated examination of the systolic function in diabetic patients is not sensitive enough for the early discovery of left ventricular dysfunction. Intentional assessment of diastolic function in patients with diabetes is advisable (278) for the early detection of left ventricular dysfunction before appearance of the clinical symptoms and for follow-up of any deterioration of cardiac status. Thus, the detection of diastolic abnormalities may be a useful indicator for the prognosis of cardiovascular mortality in diabetic subjects. A 3-year follow-up study comparing groups of diabetic patients with normal diastolic function to those with diastolic dysfunction revealed that the former group had survival rates close to those predicated, but 31% of those with diastolic dysfunction, even those who were asymptomatic, developed heart failure, and 19% died (316).

2. Animal studies

The use of animals for studying changes in cardiac contractility is particularly advantageous because of the number of parameters which can be more critically and accurately measured. In vitro heart preparations such as atrial preparations, isolated hearts, working hearts, papillary muscles and in vivo hemodynamic studies have been performed with diabetic animals. These experimental studies have been necessary in order to obtain a more clear understanding of the pathophysiology of diabetic cardiomyopathy which is characterized by a decrease in both systolic and diastolic function. This decrease in heart function can occur in diabetes mellitus independent of the presence of any other risk factors associated with cardiovascular disease (13). Detailed experiments have shown the presence of a cardiomyopathy in both chemically (alloxan/STZ) and spontaneously diabetic rats similar to that observed in diabetic patients.

Many of the studies from several independent laboratories have reported alterations in cardiac performance in diabetic animals. With all but one exception (251), all of the investigators have shown that the heart is in a depressed functional state during diabetes. The most extensive studies were carried out in the mid-1970's (251) and early 1980's (120,134) still remain landmark investigations in this area. Many parameters have been monitored to assess cardiac performance in animals during diabetes. In general, the findings with diabetic animal hearts, which demonstrate a significant depression in contractile performance of the cardiac muscle, correspond to those obtained in human diabetics. These can be subdivided into tension (force) generation and relaxation. Generally, all indices of force generation in the hearts of diabetic animals were subnormal. The ability to generate a peak amount of force

or pressure is depressed. In addition, the rate of force or pressure development was slower (119-121,123,134,136,177,318-327). These defects were translated into functional depressions in hemodynamic parameters such as aortic output, stroke volume and cardiac work and cardiac output (120,121,177,251,318,319,328). In addition, other affected indices of force generation in the hearts from diabetic animals include augmentation of: time to peak tension, time to peak shortening, and time to peak shortening velocity (134). Alterations in end-diastolic volume have been reported (120,251); however, depending probably upon the severity and duration of diabetes, the direction of the change and its relationship to pressure development was found to be variable (120,251). The change in the ability of the heart to relax during diabetes can be even more dramatic than the changes in force generation (134). The presence of diabetes appears to slow the rate of relaxation and prolongs the amount of time it takes to dissipate tension. Changes in tension generation and relaxation in papillary muscles from diabetic and control animals were demonstrated from an exceptional study by Fein and colleagues (134). When one considers various indices of relaxation affected in hearts from diabetic animals, studies have overwhelmingly shown: attenuation of peak velocity of relaxation, - dP/dt as well as -dT/dt, and augmentation of such parameters as: time to peak relaxation velocity, time for peak tension to fall by 50%, and time to peak rate of tension fall (119,120,123,134,321,322).

One factor that influences cardiac performance during diabetes is duration of diabetes. Miller (121) observed a depression in systolic pressure development, cardiac output, and aortic output of the isolated perfused working heart from diabetic rats as early as 3 days after the

induction of diabetes with an injection of alloxan. This impairment in cardiac performance could be normalized by including insulin in the perfusate or elevating perfusate glucose concentrations substantially. Thus, it was concluded that the defect in cardiac function was due to an inability of the acutely diabetic rat heart to use glucose (121). However, this was not found to be the case in the chronically diabetic animal. The defects in cardiac performance in rats made diabetic for several weeks have been shown to be corrected to some extent (120,318,319) by altering glucose delivery to the heart, but these hearts still exhibited significant functional depression in comparison to controls (120,134,119). Thus the defect in cardiac performance observed in chronically diabetic rats may not primarily be to an inability to utilize perfusate glucose. Instead, intrinsic defects in the cardiac excitation-contraction coupling process have been suggested to cause the functional impairment (91,321,329). Various studies have shown 1 month of diabetes is necessary before contractile deficiencies can be observed in hearts from chemically induced diabetic rats (119,134). In other studies the duration of diabetes is required to be 2 1/2 - 3 months before cardiac dysfunction was apparent in the chemically induced diabetic rabbit (136,137). The data from various laboratories would support the contention that the type of diabetes present does not affect the appearance of cardiac dysfunction. Insulin-dependent diabetes of a spontaneous (330,331) or chemically induced nature produces structural and/or functional changes in the heart, as previously described. Cardiac dysfunction in a chemically induced model of noninsulin diabetes has been reported (318); however, the characteristics of this diabetic model (fasting euglycemia in the presence of glucose intolerance) may instead

reflect an insulin deficient state of lesser severity rather than a true-noninsulin-dependent diabetes mellitus (NIDDM).

The severity of the diabetes mellitus may also represent an important factor in determining the expression of cardiac disease. Most studies of chemically induced diabetes have employed rather large dosages of alloxan or STZ ($\geq$ 50 mg/kg body weight) to produce highly elevated fasting blood glucose levels (> 400 mg%) (119-121,134,320). The contractile dysfunction associated with this condition has been significant as well. In contrast, Regan and co-investigators (251) have used low doses of alloxan (20 mg/kg body weight) on multiple occasions (three times at monthly intervals) in dogs to produce a mild diabetic condition. Fasting blood sugar levels are normal but glucose intolerance is observed (251). They have found lower end-diastolic volume in diabetic dogs and double the increase in end-diastolic pressure in ventricles from diabetic dogs in response to volume expansion tests (251). These findings are in opposition to those of Penpargkul and co-workers (120) using a more severe model of diabetes in rats. The conflict in results is important because of their implications regarding the mechanism responsible for the functional defects in the heart. Regan and colleagues (251) concluded that the cardiac dysfunction is caused by a reduced compliancy of the ventricular wall whereas Penpargkul and co-workers (120) concluded that their results are consistent with a contractile defect rather than a compliancy problem. Further examination of the factors responsible for the conflict is warranted; however such a difference in results is further complicated by the possibility that species may also have influenced the findings. This is more directly supported by a study by Fein et al (134,320) who found important

differences in the time-dependency of the appearance of some mechanical alterations in the heart. Alterations in myocardial glycoprotein composition have been observed in chemically induced diabetic rabbits (136) and dogs (251) but not consistently in rats (253,331). Again, these discrepancies have important implications with respect to the mechanisms responsible for the cardiomyopathy in diabetes.

Further alterations in the performance of hearts from diabetic animals are unmasked by various stimuli. The response of the heart to increasing Ca^{2+} concentrations in the perfusate difference between control and diabetic animals (177,332). In the study by Bielefield et al (177), both left ventricle pressure development and aortic output were depressed at low but not higher calcium concentrations in diabetic preparations in comparison to control preparations. Supraphysiological Ca^{2+} concentrations again revealed a depression in cardiac function (177). These results have suggested that the myocardium cannot regulate intracellular Ca^{2+} concentrations. Alterations have also been reported for the response of cardiac preparations from diabetic animals to glycosidic stimulation (324,332). Perfusion of papillary muscles with varying concentrations of ouabain resulted in a greater rise in resting tension and a larger fall in developed tension in diabetic preparations as compared to control preparations (324). This resulted in contractures at a ouabain concentration of 0.1 mM (333). Insulin treatment of the diabetic animals was found to normalize these alterations. Changes in inotropic response of the heart to other agonists has been observed. B-adrenoceptor agonist, isoproterenol, was shown to be far more potent in stimulating inotropy in cardiac muscles from control rats as opposed to diabetic rats (283).

Myocardial dysfunction was present in newborn children of diabetic mothers (333). In this regard, an interesting investigation by Jarmakami and colleagues (334) dealt with myocardial excitation-contraction coupling in the fetal hearts from diabetic rabbits. Diabetes was induced by alloxan injection in pregnant diabetic rabbits by day 14 of gestation and the fetal heart was removed 14 days later for mechanical analysis. These investigators discovered no difference in force generation between control and diabetic preparations in the presence of 1.5 mM Ca^{2+}, however, at higher perfusate Ca^{2+} concentrations, the inotropic effect was more pronounced and the toxic effect was significantly less in the fetal heart preparations from the diabetic mother as opposed to the nondiabetic mother (334). These changes in myocardial Ca^{2+} regulation appeared to be due to alterations in Ca^{2+} movements across the sarcolemmal membrane and not due to changes in the accumulation of Ca^{2+} by intracellular membrane systems (334). It should be pointed out that similar defects in myocardial regulation of Ca^{2+} flux at the sarcolemmal membrane level have been described in diabetes in adult animals (122).

3. Methods to normalize cardiac function in diabetics

The presence of significant abnormalities in cardiac performance during diabetes are thought to be related to epidemiological and autopsy findings of an increased frequency of cardiac lesions and cardiac failure in the diabetic population. This has led researchers to investigate methods of treating the cardiac dysfunction. Questions such as: (i) Can the depression in heart function during diabetes be normalized? (ii) What kinds of therapy can most effectively treat heart disease in the diabetic? The most direct approach to the problem, and the one most

frequently employed, has been to examine a correction of the hyperglycemic condition of animals results in a normalization of cardiac function. Insulin administration of diabetic animals has been the obvious method of choice in most investigations; however, other means of achieving normal glycemic status have been used to determine its effect on heart performance. Because of the presence of concomitant alterations in hormones other than insulin in the diabetic state, the normalization of such hormonal balance in the presence of diabetes has also been examined with regard to its effect on myocardial performance. Because of ethical considerations, the withdrawal and administration of diabetic therapy has been studied only in animal models. Accordingly, the effects of insulin therapy on diabetic cardiomyopathy were studied by Fein and colleagues (335). At 5 to 8 weeks after inducing diabetes in rats by STZ injection, insulin was administered to the animals by daily subcutaneous injection for 4 weeks. This treatment normalized plasma glucose concentration and corrected the changes in body and heart weight associated with the diabetic condition (335). Isometric contractile defects evident in papillary muscles from diabetic rats were corrected by the insulin treatment (335). Since insulin addition to the muscle bath of diabetic preparations did not correct the contractile deficiencies (335), it was concluded that the normalization was a result of insulin therapy.

The duration of the insulin treatment is an important factor in normalizing muscle function of diabetic rats. This however, is not correlated with blood glucose levels which are almost immediately corrected by insulin administration (335). In left ventricular papillary muscle functional studies of insulin-treated diabetic rats, a gradual

reversal of the prolongation of time to peak tension was observed (335). Insulin treatment for 10 days was sufficient to partially reverse this contractile defect. A full 4 weeks of insulin administration to the diabetic rats brought these contractile defects back to normal. The dosage of insulin was also found to be important regarding the ability of insulin therapy to reverse cardiac dysfunction. At 7 to 8 weeks after the induction of diabetes in rats by STZ injection, animals were given daily subcutaneous injections of insulin for a further 6 weeks (336). Papillary muscles were dissected from control or diabetic rats which were given doses of insulin varying from 0 to 2.5 U/day. The results demonstrated a graded recovery in muscle function depending upon the dose of insulin (336).

Several other factors are important to recognize concerning the effects of insulin therapy on cardiac performance of diabetic animals. The studies discussed above used a duration of diabetes of about 2 to 3 months (335,336). Insulin therapy also partially or totally reversed the alterations in cardiac function associated with long-term diabetes (5 to 6 months) in rats (252). However, this was not the case in mildly diabetic dogs (252). The myocardial abnormalities observed in dogs maintained in a diabetic state for 1 year could not be reversed by insulin administration (252). These findings may be qualified by longer durations of insulin treatment (252). The cardiac dysfunction which can be reversed by insulin treatment can also be prevented by insulin treatment. If, instead of administering insulin to diabetic animals which already exhibit cardiac abnormalities, insulin is given immediately after inducing diabetes, the appearance of cardiac dysfunction was prevented (327). Thus insulin therapy of diabetic animals may yield

some information regarding the factors which contribute to the cardiac performance defects. On the basis of results obtained by varying the insulin dose in diabetic animals (336,337) or administering insulin to control animals (338), it has been suggested that insulin deficiency rather than hyperglycemia may be a more important factor in cardiac dysfunction during diabetes. It is known that insulin can exert an inotropic effect in the heart which is independent of its effects on substrate supply (339). Furthermore, insulin has been shown to alter ion interactions with several myocardial subcellular organelles (91). However, the topic of the role of insulin-deficiency vs hyperglycemia in the diabetic cardiomyopathy requires further investigation before firm conclusions may be made.

Therapy other than insulin administration to diabetic rats has been instituted in an effort to reverse or prevent the depression in cardiac performance. Various methods of restoring euglycemia to insulin-deficient diabetic animals have been utilized by different laboratories. Hungarian investigators (340) compared the effects upon the myocardium of insulin therapy and sulfonylurea therapy in diabetic dogs. After injection with the diabetic agent alloxan, animals were treated with insulin, carbutamide, or glibenclamide for 3 months. The insulin was delivered subcutaneously by injection and these sulfonylurea drugs were given orally to the animals at meal time. After the treatments, there was a significant improvement in myocardial performance, ventricular compliancy, and accumulation of connective tissue in hearts from the diabetic dogs (340). No difference was observed among the three agents with respect to myocardial recovery from the diabetes-related performance defects. However, carbutamide treatment did cause a significant

elevation in arterial blood pressure in the diabetic dogs (340). Since sulfonylurea drugs can improve cardiac performance by simply including the drug in the perfusion medium and stimulating energy metabolism (319), its mechanisms of action is thought to involve both pancreatic and sites of action (319,341).

Another method used to prevent the decline in cardiac performance during diabetes was vanadate treatment. Vanadate is the oxidized form of the trace element vanadium; it has an insulin-like action in the cell (342). Vanadate, when included in the drinking water of diabetic rats, restored plasma glucose levels to control without influencing plasma insulin concentrations (343,344). The left ventricular developed pressure, + dP/dt and - dP/dt were significantly depressed in the diabetic animals. After vanadate treatment, these functional parameters were normalized in the diabetic rats. Unfortunately, side effects of vanadate may limit its clinical usefulness, at least in the near future. Since cardiac dysfunction recovered without any improvement in plasma insulin concentrations but with a dramatic improvement in blood sugar levels, this would support a role for hyperglycemia and not hypoinsulinemia in the cardiac disease. However, the validity of this contention is complicated and seriously threatened by the knowledge that the molecular mode of action of vanadate is very much like that of insulin (342).

Circulating thyroid hormone concentrations are depressed in both diabetic rats (123,345,346) and humans (347). Hypothyroid animals demonstrate a depression in cardiac pump function (348). These abnormalities in cardiac performance in the hypothyroid state are similar to those exhibited by the diabetic animal. It is possible, therefore,

that the cardiac dysfunction present in the diabetics may be due to the accompanying thyroid hormone deficiency. Several investigators have examined this possibility by administering thyroid hormone to diabetic animals. Normalization of circulating thyroid hormone levels did not restore cardiac performance (345) nor did it bring the activity of several subcellular organelles, which are associated with force generation in the heart, back to control values (123,346). Administration of pharmacological doses of thyroid hormone was found to normalize contractile protein enzymatic activity (346); however the results do not support a role for depressed circulating thyroid concentrations in the diabetic cardiomyopathy. This view was further strengthened by evidence of depressed cardiac function in animal species which maintained normal circulating thyroid hormone levels during diabetes (136,330).

Dichloroacetate (DCA), a pyruvate dehydrogenase activator, is a pharmacological agent that is effective in increasing myocardial glucose oxidation in normal and diabetic rat hearts perfused with glucose and insulin (349,350). Nicholl and co-workers (351) indicated that even in the presence of elevated levels of fatty acids, DCA dramatically stimulated glucose oxidation, and glucose oxidation in chronically diabetic rat hearts.

In their study, these investigators demonstrated that diabetic hearts perfused with palmitate plus DCA showed a marked improvement in function. Heart rate and the heart rate-peak systolic pressure product in spontaneously beating hearts, as well as left ventricular diastolic pressure and + dP/dt in paced hearts were all restored to control heart values. This study (351) emphasized that the depression of glucose

oxidation in diabetic heart may be a potentially significant factor contributing to cardiac dysfunction.

From the foregoing discussion, it is evident that cardiac performance is severely altered in diabetes. This view is supported by an extensive examination of several indices of cardiac performance which showed that mechanical function is disturbed by diabetes in both humans and animals. Systolic time intervals are abnormal and echocardiographic as well as angiographic evidence demonstrate an impairment in cardiac pump performance during diabetes in humans. These changes occur in the absence of major vessel disease. Microvascular lesions and compositional alterations in the diabetic heart have been suggested as casual mechanisms for the defects in cardiac performance. These results in humans have been corroborated by observations of contractile dysfunction in cardiac preparations from diabetic animals. The heart dysfunction occurred without large vessel arterial disease and hence, supports the existence of a primary defect in the heart muscle during diabetes. These contractile performance defects can be prevented and reversed by insulin treatment of diabetic animals. Corrections of hyperglycemic state by insulin administration or other types of therapy have proved to be a very successful means of correcting defects in cardiac performance.

E. Subcellular Defects in Diabetic Cardiomyopathy

Force generation in the heart is ultimately a cellular event and hence any alteration in the pumping ability of the heart must reflect a change in some aspect of the process of excitation or relaxation at the cellular level. To date, much data have supported a close association of the changes in cell Ca^{2+} concentrations with the contractile event in the heart. Calcium entry from the extracellular space into the

myocardial cell during the action potential elicits contraction. Free Ca^{2+} concentrations in the cytoplasm rise to about 10^{-5} M during tension generation and then return to about 10^{-7} M upon relaxation of the myocardium. Obviously, therefore, the factors which regulate Ca^{2+} homeostasis in the cell and those components in the cell which interact with Ca^{2+} ions are important to the contractile status of the heart. Several structures in the myocardial cell are ultimately involved in the control of tension generation and relaxation in the heart. The sarcolemmal membrane, the sarcoplasmic reticulum (SR), the mitochondria, and the contractile proteins are the principal subcellular organelles involved in contraction-relaxation process in the normally functioning heart. Since these organelles are important for the viability and function of the normally healthy myocardium, it is reasonable to postulate that a lesion in these organelles may be associated with the depression of cardiac function observed in many disease conditions. The possibility that the functional abnormalities associated with diabetes mellitus may result from significant defects in the viability of various organelles has been addressed by a number of independent laboratories in several countries. Lesions in various subcellular organelles have been identified and extensive investigations have been carried out to characterize the nature of such lesions. In this section, a discussion concerning the functional integrity of the contractile proteins, sarcolemma, mitochondria, and SR in cardiac tissue from diabetic animals is presented. An attempt has been made to understand how these defects may relate to the observed depression in force generation in the diabetic heart.

1. Contractile proteins

The final reaction in the sequence of subcellular events leading contraction in the heart ultimately lies with the contractile proteins. Ca^{2+} binding to troponin C protein allows myosin to interact with the actin through a complex conformational change (352). In the process, adenosine triphosphate (ATP) is hydrolyzed to adenosine diphosphate (ADP) and inorganic phosphate (Pi) by the enzyme adenosine triphosphatase (ATPase) which is located on the head region of the myosin molecule (353). The observation that the contractile protein ATPase activity could be closely correlated with cardiac muscle function (354) has allowed researchers to obtain a biochemical marker of contractile protein function which also has important relevance to muscle performance. It is possible to separate contractile proteins by various biochemical means into distinct preparations of varying purity (353). Several preparations of contractile proteins are presently examined routinely by many laboratories across the world but four fractions have achieved widespread use: myofibril, actomyosin, myosin, and heavy meromyosin. The myofibrillar preparation contains a more complete complement of contractile proteins and, therefore, may represent a closer approximation of the actual physiological setting (353). However, many experiments require a purer preparation of contractile proteins to avoid possible errors in interpretation of results. The myosin or heavy meromyosin preparations represent attempts to purify the contractile proteins to a single protein or protein unit. Each "preparation" has its own advantages and disadvantages (353) and such considerations will thus limit the value of the conclusions which are obtained.

Purified myofibrillar fractions isolated from hearts of STZ-induced, chronically diabetic rats showed depressed Ca^{2+}-stimulated

ATPase activities in comparison to those observed in control (355-357). This defect was found to be closely associated with the hyperglycemic status of diabetic animals. If this condition was corrected by chronic insulin administration, Ca^{2+}-stimulated ATPase activity returned to normal (357). The depression in Ca^{2+}-stimulated ATPase activity of cardiac myofibrils from diabetic animals was observed over a full range of physiologically relevant Ca^{2+} concentrations (355,358). Myofibrillar Mg^{2+}-dependent ATPase activity in diabetic animals also exhibited a similar loss of activity in comparison to control preparations and this defect was eliminated after chronic administration of insulin to diabetic animals (356). Studies with alloxan diabetic rabbits (136,358) have also revealed depressed myofibrillar Ca^{2+}-stimulated ATPase activity with subsequent normalization of function upon insulin administration.

The mechanism responsible for the defect in myofibrillar ATPase activity has been difficult to identify but several possibilities have been put forward. Gross changes in myofibrillar structure are unlikely to be present since sodium dodecyl sulfate (SDS) electrophoretic analysis of myofibrillar samples revealed no significant differences between control and diabetic preparations (359). Phosphorylation of specific proteins in the myofibrillar fraction is known to influence myofibrillar Ca^{2+}-ATPase activity. However, this is unlikely to be responsible for the changes observed in diabetic myofibrillar ATPase activities because similar levels of in vitro phosphorylation in both control and diabetic cardiac myofibrillar fractions has been observed (359). Certainly it is not a direct effect of the removal of insulin from the circulation of diabetic animals, since insulin had no effect on myofibrillar function

(359). The most likely explanation for the change in myofibrillar ATPase activity during diabetes may involve a localized modification in structural conformation at or near the active site of enzymatic activity; this may involve sulfhydryl residues. In one study myofibrillar fractions from diabetic rat heart exhibited a different reactivity to sulfhydryl group modifiers (356); such alterations in the diabetic animals could be reversed by chronic insulin administration. Thus, these experiments and others support the contention that conformational changes near the active site of ATPase activity exist in diabetic myofibrils and may be an important factor in the observed enzymatic alterations (355,356). Nonetheless, the significance of the change in myofibrillar Ca^{2+}-ATPase activity lies in its close relationship with force generation in the heart (352). Elevated ATPase activity in myofibrils has been associated with an increase in contractile force, and conversely, reduced ATPase activity has been associated with a depression in contractility in disease or failing hearts (353). The depression in myofibrillar ATPase activity in hearts from diabetic animals may, therefore, be related to the contractile dysfunction.

Dillman (361) was the first to report detailed evidence of contractile protein dysfunction in the heart during diabetes mellitus. Ca^{2+}-activated ATPase activities of actomyosin and myosin were observed to be significantly depressed in hearts from diabetic animals. These results have been confirmed by several other investigators (138,338,357,358,362,363). The existence of changes in myosin K^{+}-EDTA ATPase activity is controversial but caloric considerations were found not to be a factor for the observed depression in Ca^{2+}-ATPase activity (135). The alterations in ATPase activity were dependent upon the

duration of the diabetic condition and were present irrespective of the sex of the animal. Further evidence supporting a close association of the diabetic condition and the myosin defects was provided by the observation that such defects could be corrected if diabetic animals were corrected by chronic insulin treatment of the diabetic animals (135,361,362). It was also shown that the accompanying hypothyroid status of diabetic animals was not a primary factor responsible for the depression in myosin Ca^{2+}-ATPase activity (135,346,363). The mechanism responsible for this lesion in myosin and actomyosin ATPase activity in hearts from diabetic animals was examined in a study in which SDS gel electrophoresis and isoelectric focusing failed to reveal any differences in gross protein structure (135). However, pyrophosphate gel electrophoresis of myosin isoenzyme components from control and diabetic rat hearts revealed significant mobility changes (361). The V_1, or high mobility component, is predominant whereas V_3, the lowest mobility myosin component, exists in relatively small amounts in control rat myosin samples. This pattern was reversed, however, in the myosin samples prepared from diabetic rat hearts; V_3 fraction was the predominant component whereas the V_1 component was very low in content. The observed changes in the myosin isoenzyme distribution in experimental preparations could be reversed if the diabetic animals were treated with insulin (135,361).

The significance of changes in the myosin isoenzyme distribution lies in the correlation with cardiac function. A predominance of the V_1 component of myosin has been correlated with high activity of myosin Ca^{2+}-ATPase enzyme and maximal shortening velocity in cardiac muscle preparations (364,365). Conversely, a predominance of the V_3

component has been associated with lower myosin Ca^{2+}-ATPase activity and reduced shortening velocity in cardiac muscle preparations. The depression in actomyosin and myosin Ca^{2+}-ATPase activity in diabetic rat heart may thus be the result of this observed change in isozyme distribution. Since different isozymic forms of myosin are under genetic control (366), translational alterations appear in the heart during diabetes. Work in this area has confirmed that alterations in the translational activity of specific messenger ribonucleic acids are present in diabetic rat hearts. The isozyme forms of myosin, V_1 and V_3, are under the regulation of two distinctly different mRNAs; translation products for these mRNAs can be influenced by insulin and thyroid hormone (366). When closely examined, it was discovered that two of the mRNA species which were altered in translation during diabetes were exclusively regulated by insulin whereas the majority of the other alterations in translation products were regulated by both insulin and thyroid hormones.

2. Sarcoplasmic reticulum changes in diabetes

One of the main functions of the SR membrane system is to release Ca^{2+} from its stores into the cytoplasm to support contraction and to take up Ca^{2+} into its cisternae from the cytoplasm to effect relaxation. It is evident that a spectrum in the relative contribution of the SR to the Ca^{2+} transient exists with respect to species as rat myocardium receives the largest contribution and rabbit heart the least (367). The SR network borders the myofibrillar contractile apparatus as well as sarcolemma and the sarcolemmal T-tubule system. The energy-dependent Ca^{2+} uptake process of the SR depends on the function of the Ca^{2+}-stimulated ATPase which is believed to represent 50%-90% of the total protein content of the SR (368). The Ca^{2+} affinity (Km of 0.5

umol/L) and Vmax of Ca^{2+}-ATPase is sufficiently high to support the concept that the activity of this enzyme is the primary determinant of the rate of fall of the Ca^{2+} transient (369) in the cardiac muscle. Experimentally, it has been convenient to measure ATP-dependent movements of Ca^{2+} uptake in the absence or presence of a precipitating anion, like oxalate. Oxalate will precipitate free Ca^{2+} which accumulates in the SR membrane vesicle during the transport of Ca^{2+}; this will stabilize intravesicular-free Ca^{2+}-binding experiments, the high intravesicular free Ca^{2+} levels would act as a feedback regulator of the Ca^{2+} pump and inhibit its activity. Therefore, the presence of oxalate in the assay medium allows for the measurement of a closer representation of the absolute capacity and rate of Ca^{2+} transport by the SR. Quantitation of Ca^{2+} flux is not the only measurement of SR function which can be determined biochemically. ATP hydrolysis by the Ca^{2+} pump protein can be measured and is expressed as Ca^{2+}-stimulated ATPase activity (359).

Since cardiac muscle from diabetic animals exhibits a slower rate of relaxation than that of controls, it has been hypothesized that an abnormality in the removal of Ca^{2+} from the cytoplasm after contraction may be present. Investigators from New York (370) were first to identify a depression in the Ca^{2+}-accumulating capacity of purified SR membrane vesicles isolated from hearts of diabetic animals. This observation was confirmed and the conclusions extended by several other investigators (123,357,371,372). Not only is Ca^{2+} uptake depressed in SR, studies have also shown a depression in Ca^{2+} binding (123), when examined as a function of free Ca^{2+} concentrations. Affected SR Ca^{2+} transport in the diabetic heart has been associated with concomitant defects in

Ca^{2+}-stimulated ATPase activity (123,357,371). SR Mg^{2+}-ATPase activity in diabetic rat heart has also been shown to be depressed (370) or unaffected (123). Nonetheless, results from different laboratories are consistent with the interpretation that a lesion in the SR Ca^{2+} transport capacity is present in the myocardium from diabetic animals. This defect appears to be a direct result of the diabetes since it becomes evident under chronic conditions; the defect in SR Ca^{2+} transport is gradual in onset, appearing 2 weeks after the diabetic condition (123). Insulin treatment of diabetic animals can reverse the lesion in Ca^{2+}-stimulated ATPase activity (123) and Ca^{2+} uptake by the SR (123,372). Although a direct action of insulin on SR Ca^{2+} transport has been reported (373), it is not clear whether this effect actually plays any role in the defects observed in the insulin-deficient diabetic state.

It has been suggested that the hypothyroid state of the diabetic animals could be responsible for the defect in SR function. In this regard, it should be noted that hypothyroidism has been shown to cause a depression in cardiac SR Ca^{2+} transport (374). However, treatment of diabetic animals with thyroxine to restore circulating thyroid hormone levels did not abolish the defect in cardiac SR Ca^{2+} uptake activity (123). Thus, it was concluded that the hypothyroid state of the diabetic animals was not responsible for the lesion. Furthermore, gross alteration in SR protein composition were not apparent during diabetes (123). Although some changes in lipid composition of the microsomal fraction were evident, these alterations were not dramatic and were thought to be unlikely to fully explain the membrane dysfunction. Regulation of Ca^{2+} transport by various phosphorylation mechanisms did

not appear to be altered in in vitro measurements with cardiac SR membranes from diabetic animals (375).

It has been suggested that the accumulation of lipids in the myocardium which occurs during diabetes (376) may be involved in the defect in SR function. In particular, long-chain acylcarnitines like palmityl-carnitine are known to accumulate in the hearts of diabetic animals (376) and such compounds can inhibit the function of the SR (371,377,378). The inhibition of control SR Ca^{2+} transport by long-chain acyl-carnitines was greater than that observed in diabetic preparations (371); thus, it was suggested that some inhibition of SR function in the diabetic preparations may be due to high endogenous levels of these lipid moieties. However, all these parameters were normalized in diabetic animals but cardiac performance remained depressed. This would suggest an important dissociation of SR function from cardiac function and imply that other factors besides lesions in the SR membrane system may be responsible for the diabetic cardiomyopathy. This may be a premature conclusion because other investigators have demonstrated that treatment of diabetic animals with carnitine normalized cardiac performance (379) and carnitine treatment was found to prevent the diabetes-induced changes in the sarcoplasmic reticular Ca^{2+} pump system (380). In addition, recent studies by Katagiri et al (380a) with STZ rats demonstrated SR depression of Ca^{2+}-ATPase activity as early as one week before the appearance of fine ultrastructural alterations.

3. Mitochondria

Mitochondria have the capacity to accumulate large amounts of Ca^{2+} and occupy a high volume of the myocardial cell, it was assumed that these organelles could participate in regulating force generation.

However, this hypothesis was discounted because of two experimental observations. First, Ca^{2+} uptake by the mitochondria was too slow to support beat to beat force generation and, second, the affinity of mitochondria for Ca^{2+} was far to low for mitochondrial Ca^{2+} transport to be active in the micromolar Ca^{2+} levels which are present during the excitation-contraction coupling process in the heart (381). The current view of Ca^{2+} accumulation by mitochondria in the heart is that it may act as a sink or buffer for Ca^{2+} under pathological conditions (382-384). When Ca^{2+} entry into the heart is excessive, cytoplasmic Ca^{2+} concentrations rise and when this rise is too high, mitochondria will begin to accumulate Ca^{2+} in an effort to buffer this excessive overload of Ca^{2+}. If the Ca^{2+} in the cytoplasm is not controlled, the heart will lose its ability to relax, arrhythmias could develop, structural damage can occur, and lysosomal enzymes may be released which can be especially destructive to the myocardial cell (384,385). Thus, mitochondria can act as a potent and important buffering component of the cytoplasmic Ca^{2+} levels in the heart. Therefore, their role as a Ca^{2+} sink is particularly crucial for preventing myocardial cell damage during pathological conditions.

It has been hypothesized that chronic diabetes could influence mitochondrial performance and this dysfunction may be related to the diabetic cardiomyopathy, since alterations in mitochondrial morphology had been well documented in the past (228,229,231).

Much work has been directed to an examination of oxidative metabolism in mitochondria from hearts of diabetic animals. It was Goranson and Erulkan (386) who first suggested the presence of a defect in mitochondrial oxidative phosphorylative activity in the heart during

acute diabetes (387-390). 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 (387,388,391) 24 to 48 hours after alloxan injection. It was found a decreased synthesis of ATP and ADP in the presence of glucose, fructose and lactate. Chen and Ianuzzo (392) also 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. Significant reductions in mitochondrial oxygen consumption and respiratory control index values were demonstrated in diabetic hearts (388); these defects were normalized by insulin administration to alloxan-diabetic rats. However, it should be pointed out that the vast majority of the past work relating to the oxidative metabolism in mitochondria from diabetic animal hearts has been done using an acute model of chemically induced diabetes (387-390). Because of the acute nature of the diabetic condition, limitations arise with respect to their relevance to the chronic physiological diabetic condition. Subsequent investigations have documented significant defects in the respiratory capacity of mitochondria isolated from hearts of genetically diabetic and STZ- and alloxan-diabetic rats (239,388,393,394). Pierce and Dhalla (394) reported that mitochondria isolated from ventricular tissue by differential centrifugation from 8-week STZ-induced diabetic rats had depressed state 3 respiration, oxidative phosphorylation rate, respiratory control index and depressed Mg^{2+}-dependent ATPase

activities. These changes were partially reversed upon 2 weeks of insulin and fully reversible after 4 weeks of insulin therapy. State 4 respiration was normal during diabetes (239,394). In addition, the ADP to O ratio was depressed only in the early stages of chemically induced diabetes (~ 4 weeks and later) (239,388,393,394). Overall, the depression in mitochondrial oxidative phosphorylation has been shown to become more severe as the duration of the diabetes lengthens (394) and can be normalized by daily insulin treatment of diabetic animals (394).

Many investigators have reported a significant depression in high-energy phosphate content in hearts from diabetic animals. These investigators (121,322,395-399) have reported that ATP content of hearts from diabetic animals is reduced in comparison to controls with depressed ATP synthesis rate in the heart during diabetes (319,402). In addition, phosphocreatine metabolism also appears to be abnormal in the heart during diabetes (398) Since the creatine kinase isoenzymes, phosphocreatine, ATP and ADP (the phosphocreatine energy shuttle) play an important role in the process of energy production and utilization in both cardiac and skeletal muscle (400,401), the impairment of the system can be seen to result in deterioration of contractile function (402). Savabi and Kirsch (403) reported a reduction in various creatine kinase (CK) isoenzymes, high-energy phosphates and mitochondrial oxidative phosphorylation in STZ-diabetic rat hearts. These changes reached their maximum level after 4 weeks of diabetes and stayed constant thereafter. All of these diabetic related alterations were reversible by 4 weeks of insulin treatment. Other investigators (404) also showed that diabetes mellitus in the STZ-diabetic rat resulted in a reduction of total CK activity and a redistribution of CK isoenzymes in the heart.

Furthermore, a decrease in CK-M mRNA was found which was consistent with the observed decrease in CK activity; chronic insulin administration reversed these changes.

In addition to some of the defects observed in mitochondrial respiratory activity, and phosphocreatine energy shuttle, mitochondrial Ca^{2+} uptake capacity has been shown to be significantly depressed in hearts from diabetic animals in comparison to control preparations (394). This depression was directly attributable to the diabetic condition since the defect was reversible by insulin administration to the diabetic animals. The depression in mitochondrial Ca^{2+} uptake activity was not seen at low concentrations of calcium and was not associated with any changes in Ca^{2+} binding activity. Only the high Ca^{2+} concentrations revealed a lesion in the mitochondrial Ca^{2+} uptake capacity in the diabetic preparations (394). The results with Ca^{2+} transport by mitochondria from diabetic animals may be interpreted to suggest a defect in the absolute Ca^{2+}-accumulating capacity by these organelles. The observations that Ca^{2+} binding was unaffected and Ca^{2+} uptake was altered only at relatively high Ca^{2+} concentrations is indicative that the diabetic mitochondria may have been unable to maintain large Ca^{2+} concentration gradients across their membranes.

It should be pointed out that previous studies from various laboratories showing derangements in mitochondrial respiration and Ca^{2+} uptake in the diabetic heart were all carried out by using isolated subcellular fractions, and represent data pooled from a mixed population of cells. In addition, most reports indicated that impairments in function of mitochondria were detectable four weeks or longer after the STZ injection (394). In a recent study by Tanaka et al (405), Ca^{2+}

uptake by mitochondria, measured in situ in permeabilized cardiomyocytes of STZ-diabetic rats, was significantly decreased compared to that of control rats, regardless of the extramitochondrial (cytosolic) Ca^{2+} concentration. Impairments were also observed in myocyte respiration, mitochondrial membrane potential. These dysfunctions were observed as early as 3 weeks after STZ administration and were reversible by insulin treatment. The exact role of changes in mitochondrial Ca^{2+} uptake in diabetic heart is not clear because disturbance in Ca^{2+} homeostasis of the heart cell was indicated by a decreased content of rapidly exchangeable $[^{45}Ca]^{2+}$ of the myocyte (405). It is believed that the latter consists predominantly of Ca^{2+} in the SR (406,407). The possibility that defects in mitochondrial Ca^{2+} transport may be secondary to impairments in SR Ca^{2+} pump activity, heart muscle contractility, and heart muscle relaxation in diabetic animals (134,359) needs to be explored further.

4. Sarcolemmal membrane

It is now well established that cardiac contraction is initiated when an action potential depolarizes the cell. Sarcolemma plays an important role in the generation and maintenance of transmembrane gradients of Na+, K+ and Ca^{2+}, which are essential for cardiac cell excitability. Operating together, cardiac sarcolemmal membrane-bound ion channels, ion-exchange systems and cation pumps contribute to the regulation of membrane potential and the cardiac excitation-contraction coupling process (408). Rapid Ca^{2+} influx is achieved through opening of the voltage-sensitive Ca^{2+} channels in the plasma membrane, whereas triggering the Ca^{2+}-induced Ca^{2+} release depends critically on Ca^{2+} stores in the SR (408). In cardiac cells, two types of Ca^{2+} channels,

namely L-type and T-type have been identified (409,410). The L-type channel is the major pathway for voltage gated Ca^{2+} entry into mammalian cardiac cells leading to excitation-contraction coupling and intracellular Ca^{2+} transients (411). Experimental findings indicate that influx of Ca^{2+} via L-type channels can provide an adequate source of Ca^{2+} to trigger the release of Ca^{2+} from the SR.

The functional status of Ca^{2+} channels in the cell can be monitored by determining the specific binding of Ca^{2+} antagonists (409). Nishio et al (413) was the first group to report on a change in voltage-sensitive Ca^{2+} channels of cardiac muscle crude membranes isolated from STZ-induced diabetic rats. They observed a 64% increase in the Bmax of [^{3}H]-PN200-110 binding sites compared to control rats without any difference in Kd; this increase in binding was found in both 6 week and 12 week STZ-diabetic rats. Two weeks STZ injection followed by an 8-week intensive insulin treatment normalized the increase in [^{3}H]-PN200-110 binding in STZ-diabetic rats to control levels. Furthermore, [^{3}H]-PN200-110 binding to control cardiac membranes was dose-dependently inhibited in the presence of verapamil but that was not the case in cardiac membranes isolated from STZ-diabetic rats.

A most recent study by Gotzsche et al (414) found unchanged receptor affinities in Ca^{2+} channels in STZ-diabetic rats. However, in 4 and 7 day STZ-diabetic rats, Bmax was depressed by about 50% compared to controls, but by 6 months, Bmax was actually increased compared to controls. Insulin treatment restored Ca^{2+} channel Bmax in diabetic animals. In contrast to these two studies, Lee et al (415) reported a decrease in both ^{3}H-nitrendipine receptor number and Kd value in STZ-diabetic rat heart crude membranes that began 3 weeks after STZ

administration. Administration of insulin to diabetic rats for 3 weeks normalized changes in both Kd and Bmax values. These results are thus at variance with the previous studies (412,413). It is postulated that such differences in results appear to be due to the stage and intensity of diabetes and it is possible that changes in dihydropyridine binding sites are biphasic in nature. This view is supported by the fact that an increase in the density of ^{3}H-nitrendipine binding sites in heart membranes was seen in younger genetically cardiomyopathic hamsters (416,417), whereas no change was observed in older animals (418). Differences in the results of these studies (412-414) was also explained by the differences in experimental conditions employed for the preparation of membranes, and not necessarily due to differences in the ligands employed.

The decrease in density of ^{3}H-nitrendipine binding with diabetic myocardial membranes has been suggestive of a decease in the number of voltage sensitive Ca^{2+} channels of the L-type in sarcolemma. This results in reduced trigger Ca^{2+} from the SR stores. A decrease in Ca^{2+}-influx involving Ca^{2+} channels would then serve to amplify the adverse effects of impaired SR pump (123,370) and reduced myofibrillar ATPase activities (91,135) on contractile force development in diabetic cardiomyopathy. Lee et al (415) also observed that absolute values for contractile force development in the isolated heart preparations at different concentrations of Ca^{2+} were less than those for the control preparations. It was noted, however, that the % increase in contractile force upon increasing the perfusate Ca^{2+} was higher in diabetic hearts. In fact, increased sensitivity of diabetic heart to Ca^{2+} with respect to contractile force development has also been reported by other

investigators (177). This change could likely be due to an increased affinity of Ca^{2+} channels in the diabetic sarcolemma (415). This view would be consistent with the observation that verapamil exerted more depression of contractile force in the diabetic heart in comparison to the control. Report of increased affinity of Ca^{2+} channels could also provide evidence to explain the occurrence of intracellular Ca^{2+} overload and subsequent myocardial cell damage in the diabetic heart (91). Overall, dramatic alterations in myocardial metabolism as a result of insulin deficiency and elevated levels of plasma glucose in diabetic animals can thus be seen to account for the observed changes in sarcolemmal Ca^{2+} channels. Such changes may also occur due to long chain acyl derivatives which can accumulate in the diabetic heart and known to exert dramatic actions on membranes (371,419-421).

The minute-to-minute maintenance of heart rate and cardiac output depends to a large extent on the responsiveness of the heart to the autonomic nervous system. In addition to the changes discussed so far in contractile machinery, sarcoplasmic reticulum, mitochondria and Ca^{2+} channels, there is much evidence for changes in cardiac autonomic receptor function and/or numbers following STZ/alloxan treatment. Savares and Berkowitz (422) were the first to report a decrease (28%) in the number of cardiac ß-adrenoceptors 8 weeks after STZ treatment with no change in ß-adrenoceptor affinity. It was suggested that this reduction in receptor number might have contributed to the bradycardia seen in the diabetic animals (422). Since that time, there have been several reports of diminished cardiac ß-adrenoceptor numbers, with no change in affinity, in cardiac tissue taken from rats 2 to 10 weeks after treatment with STZ (55,321,423-428) or alloxan (429). It is likely that these changes

develop gradually, because Gotzsche (178) found no change in cardiac ß-adrenoceptor numbers 8 days after STZ treatment, and Latifpour and McNeill (430) found a small, nonsignificant decrease in ß-adrenoceptor numbers 3 months after STZ treatment but a significant decrease after 6 months. Since Gotzsche et al (414) most recently reported down-regulation of ß-receptors as early as 4 days after STZ administration, there appears to be no consistency with regard to the time of onset of changes in ß-adrenoceptor numbers in different studies. Neither is there a clear relationship between these changes and the time of onset of bradycardia, which may occur within 4 days of STZ treatment (431). Nonetheless, alterations in ß-adrenoceptor numbers appear to be relatively restricted to cardiac tissue because no change occurred in the ß-adrenoceptor populations of lung membranes from rats treated with STZ (430). In a study using refinement of earlier approaches, Kashiwagi et al (432) measured concentrations of cell surface and total cell ß-adrenoceptors of cardiac myocytes 10 weeks after STZ-treatment. Although there was a 41% reduction in cell surface-binding sites, there was no difference between STZ-treated and control rats in total cell receptor concentrations, suggesting abnormalities in ß-adrenoceptor recycling.

In addition to effects on ß-adrenoceptor number, chemically-induced diabetes by either alloxan/STZ treatment may also lead to uncoupling of the ß-adrenoceptor from second messenger systems. Gotzsche (178) found that, in cardiac tissue taken 8 days after treatment with STZ, ß-adrenoceptor number was unaltered, but cyclic adenosine monophosphate (cAMP) accumulation in response to the ß-adrenoceptor agonist, isoprenaline, was diminished. In contrast, Atkins et al (425) found that cAMP accumulation in response to isoprenaline was normal in cardiac

tissue taken from rats treated 2 weeks previously with STZ, but was impaired 4 weeks after STZ treatment. In that study, there was a similar reduction in ß-adrenoceptor number at both times. Because of no change in basal cAMP production (178,425,427) was found following STZ treatment, it was suggested that the coupling of ß-adrenoceptors to adenylate cyclase may be abnormal. This might have been due to changes in regulatory guanosine triphosphate binding proteins (427), because there was evidence for an increase in Gi proteins (i.e. those that inhibit adenylate cyclase) in cardiac tissue from STZ-treated rats. Isolated papillary muscle, isolated perfused hearts, and ventricular tissue and atria from STZ-treated rats and alloxan-treated rats also showed attenuated contractile responses to isoproterenol and noradrenaline (55,321,433-436). In contrast to these studies, Austin and Chess-Williams (437) found enhanced responsiveness of isolated papillary muscles and atria from STZ-treated rats to isoprenaline and forskolin and an increase in ß-adrenoceptor number. The major difference in methodology was that Austin and Chess-Wiliams used female rats in their study while male rats were used in all of the other studies with the exceptions of those of Gotzsche (178) and Goyal et al (436). Because sex differences are known to occur in various cardiovascular responses mediated by the noradrenergic system (438), it is possible that STZ treatment has different effects in male and female rats.

There are several reasons why cardiac adrenoceptor function may be altered following STZ treatment. It is possible that enhanced turnover of catecholamines in the myocardium could contribute to adrenoceptor down-regulation. Ganguly et al (439,440) made detailed studies of noradrenaline metabolism in cardiac tissue from STZ-treated rats. They

found that not only was cardiac noradrenaline content increased following STZ-treatment but also noradrenaline turnover, synthesis, and release were increased. The difference in noradrenaline turnover between control and STZ-treated rats was abolished following treatment with the ganglion blocker, pentalinium, indicating that the increase in noradrenaline turnover in STZ-treated rats may have been due to enhanced sympathetic nerve activity. The results of Ganguly et al (439) are in agreement with those of Lucas and Qirbi (441) who also found enhanced noradrenaline turnover in the ventricle of STZ-treated rats but disagree with those of Yoshida et al (442,443) who obtained evidence for reduced noradrenaline turnover in hearts of STZ-treated rats. Thus, there remains a controversy about the effects of STZ or alloxan treatments on noradrenaline turnover. Furthermore, when cardiac noradrenaline content and ß-adrenoceptor numbers were measured in the same study (425), no change in the former was found at a time when the latter was reduced. Hypothyroidism following STZ/alloxan treatment also has been suggested to contribute to the diminished number of cardiac ß-adrenoceptors (424,433), because rats that had been thyroidectomized prior to STZ treatment developed no further reduction in ß-adrenoceptor numbers after STZ treatment (424), and administration of T4 to intact rats following STZ treatment prevented the decrease in cardiac ß-adrenoceptor numbers (424). Karasu et al (433) using thyroidectomized alloxan-induced diabetic rats reported that insulin administration to these animals did not reverse the diabetic-induced changes in spontaneously beating rat atria and suggested that the thyroid hormones are needed for insulin to normalize the alterations in diabetic heart tissue. The role of hypothyroidism is, however, contentious because reductions in plasma thyroid hormone levels

are not temporally related to changes in cardiac ß-adrenoceptors, and an insulin regimen that reversed the effects of STZ/alloxan on cardiac ß-adrenoceptors had no effect on plasma thyroid hormone levels (427). Furthermore, Goyal et al (436) found that T3 treatment, at a dose that prevented the development of bradycardia, did not prevent the impairment in atrial chronotropic or inotropic responses to isoprenaline following STZ treatment.

Although there is some consistency among studies in showing reductions in ß-adrenoceptor numbers and impaired coupling to second messenger systems and contractile responses, the time course of these changes and the relationship between them are not clear. In order to investigate $[Ca^{2+}]i$ in response to ß-adrenergic stimulation, Yu et al (444) used isolated myocytes to clarify the mechanisms involved in diabetes-induced ß-adrenergic signal transduction. The results of their elegant studies suggested that in addition to alterations in ß-adrenoceptor function,there were post receptor defects in diabetic myocardium that could likely impair $[Ca^{2+}]i$ in diabetic myocardium.

A reduction in cardiac alpha-adrenoceptor number with no changes in affinity occurs following induction of the diabetes (321,423,426,430,445). The responsiveness of STZ-treated rats to alpha-adrenoceptor agents may also depend on the tissue used because, although contractility of isolated papillary muscle to the $alpha_1$-adrenoceptor agonist, methoxamine, was attenuated following STZ treatment (321), the $alpha_1$-adrenoceptor-mediated chronotropic and inotropic responses of atria or ventricles isolated from STZ-treated rats were enhanced (435,436,446-448). Discrepancies between the effects of STZ treatment on alpha-adrenoceptor numbers and responsiveness to alpha-adrenoceptor

agonists may arise because of changes in the activity of second messenger system. It was found that atrial calcium turnover in response to alpha-adrenoceptor stimulation was enhanced (447), and there were increased atrial and ventricular contractile responses to calcium (435) following STZ treatment. Furthermore, inositol (1,4,5)-triphosphate production in response to noradrenaline was enhanced in ventricular tissue isolated from STZ-treated rats (448). In a study by Jackson et al (447), phenylephrine in the presence or absence of the ß-adrenoceptor antagonist, timolol, was used to assess chronotropic responses. These authors observed that the response to phenylephrine alone was inhibited by timolol to a lesser extent in tissue from STZ-treated rats than in control animals. It was suggested that, whereas alpha-adrenoceptor stimulation is normally of little importance in the control of heart rate this may become increasingly important due to a diminished contribution from ß-adrenoceptor stimulation following STZ-treatment. Changes in myocardial eicosanoid production has been suggested to underline altered alpha-adrenoceptor function in STZ-treated rats (449). In addition to changes in autonomic neuronal function, prostaglandin production and hypothyroidism, there are a number of other factors that may be responsible for the abnormalities in cardiac alpha-adrenoceptor mechanisms. These appear to include changes in cardiac membrane fluidity, and/or nonenzymatic glycosylation of proteins that occur during hyperglycemia (449). Impairment of cardiac $alpha_1$-adrenoceptor signalling has also been shown to be closely associated with the diabetic state, and may be linked, at least in part, with abnormal activation of cardiac protein kinase C (445).

Muscarinic cholinergic receptors in the ventricular tissue of the

heart have been found to be altered during long-term diabetes (430); this effect was found to be dependent upon the duration of diabetes (423,430). As was the case with ß-adrenergic receptors, muscarinic receptor density was depressed in hearts from diabetic animals but affinity was unaffected (430). A similar observation has been reported for atrial tissue from diabetic rats; however, the decrease in muscarinic receptor density was found at earlier times after the induction of the diabetic state (450). These changes in receptor density may partially explain changes in the sensitivity of diabetic rat hearts to cholinergic stimulation (451).

The Na^+-K^+ pump, measured biochemically as Na^+-K^+-ATPase activity, is localized in the cardiac sarcolemma and produces an uphill transport of sodium and potassium ions at the expense of ATP hydrolysis (452-454). Regulation of intracellular homeostasis of Na^+ and K^+ is vital for normal cardiac cell function as homeostasis of both these ions is necessary to maintain electrical properties of the myocardium. The concept that a rise in intracellular Na^+ concentration via inhibition of the Na^+-K^+ pump enzyme contributes to sarcolemmal Ca^{2+} influx by activating the sarcolemmal Na^+-Ca^{2+} exchange system was emphasized by Schwartz et al (453). Thus, any change in the operation of sarcolemmal Na^+-K^+ ATPase activity conceivably could alter Ca^{2+} movements in the cell and thus may be seen to modify cardiac contractile function. Several studies have presented evidence which suggest significant dysfunction in the cardiac plasma membrane at the site of Na^+-K^+ ATPase. Onji and Liu (455) were the first to demonstrate that diabetes could be associated with a defect in the Na^+-K^+ ATPase enzyme system. This study showed that K^+-stimulated para-nitrophenyl phosphatase (K^+-pNPPase) activity in diabetic myocytes was significantly depressed

in comparison to control values. Since K^+-pNPPase activity is thought to represent the dephosphorylation step of the Na^+-K^+ ATPase enzyme, this would suggest that a defect in Na^+-pumping across the sarcolemma may exist in diabetic animals. Pierce and Dhalla (158) were the first to isolate a purified preparations of cardiac sarcolemma from diabetic animals in order to more closely examine the integrity of enzymes located in the plasma membrane; both oubain-sensitive K^+-pNPPase and Na^+-K^+ ATPase activities were significantly depressed in diabetic preparations in comparison to controls. The depression in Na^+-K^+ ATPase activity correlated well with and may explain the depression of the Na^+-pump activity observed in ventricular slices from diabetic rats (324); this change was reversible by insulin. Subsequent studies have confirmed depressions in Na^+-K^+ ATPase in experimental diabetes (456-459). Kjeldsen et al (460) reported that a depression in the number of ouabain cardiac binding sites were present with a concomitant decrease in Na^+-K^+ pump concentration in STZ-rats. Depressed ouabain binding in cardiac tissue during diabetes and attenuated inotropic response to ouabain have also been observed (461). Efforts have been made by many investigators to elucidate the physiological and pathophysiological role of the sarcolemmal Na^+-Ca^{2+} exchange system in the heart since the discovery of Na^+-dependent Ca^{2+} transport (462,463). The kinetic parameters suggest that the sarcolemmal Na^+-Ca^{2+} exchange system is capable of rapid movements of Ca^{2+} in and out of the myocardial cell. In electrophysiological studies, SR Ca^{2+} release was demonstrated to occur in response to graded sarcolemmal Ca^{2+} influx (464), whereas the Na^+-Ca^{2+} exchange mechanism has been suggested to cause transsarcolemmal Ca^{2+}-influx subsequent to a rapid influx of Na^+

through the fast Na^+ channels (465). On the other hand, Bridge et al (466) have provided evidence to suggest that Ca^{2+} entering the heart cell during excitation is extruded by the Na^+-Ca^{2+} exchanger system. Thus sarcolemmal Na^+-Ca^{2+} exchange may be important in mediating both influx and efflux of Ca^{2+}, and thus may be implicated in contractile function of the heart. The Ca^{2+}-stimulated ATPase localized in the cardiac sarcolemma and described biochemically as Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase is generally believed to be involved in transsarcolemmal Ca^{2+} extrusion (467-469). The sarcolemmal Ca^{2+}-pump is under the control of phosphorylation/dephosphorylation reactions, mediated by Ca^{2+}-calmodulin-dependent and cAMP-dependent protein kinases (470,471). This mechanism of regulation may be important in balancing the increased influx of Ca^{2+} due to beta-adrenoceptor activation of the slow Ca^{2+} channels. Sarcolemmal Na^+-Ca^{2+} exchange and Ca^{2+} pump activities (measured biochemically as Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+} uptake or accumulation) have been shown to be developed in chronic diabetes and normalized by insulin administration (456-458,472).

One of the mechanisms for the movement of sodium into the myocardium is through sodium-hydrogen exchange (473). This ion transport system has been identified in myocardial cells (390) and in isolated, cardiac sarcolemmal membrane vesicles (474). The transporter is electroneutral and sensitive to various cations (474). In addition to its role in controlling sodium entry into the myocardial cell, the accompanying hydrogen efflux may also play a role in the steady-state maintenance of intracellular pH. A study by Lagadic-Gossmann et al (475) observed no difference between the steady-state pHi values recorded from

diabetic or normal papillary muscle. On the other hand, the amplitude of the acidification induced by withdrawal of NH_4+ was markedly increased in diabetic papillary muscles vs control and there was a marked slowing down of the recovery from acidosis in diabetics. These early findings suggested that diabetes is associated with a change in the activity of the amiloride-sensitive Na^+-H^+ exchange. In isolated sarcolemmal vesicles from STZ-diabetic rats, Pierce et al (458) noted a striking depression (67%) in cardiac sarcolemmal Na^+-H+ exchange compared to control. These results represented the first observation of an alteration in Na^+-H+ exchange in cardiac sarcolemma during a diseased state. These results suggest that intracellular pH and $[Na]^+$ may also be disturbed in the myocardium during diabetes. Altered Na^+-H^+ exchange activities in diabetic rat hearts can have a profound effect on pH_i and functional recovery in the early stages of reperfusion from ischemic challenge (476).

5. Cardiac membranes and electrophysiological activities in diabetes

Lipids, proteins and carbohydrates are three major important components that constitute the cell membrane. These three components have received some attention as possible determinants of cardiomyopathy during diabetes mellitus. A change in the composition of the sarcolemmal membrane, or other membrane systems, has two important ramifications. A change in the membrane microenvironment and its biophysical properties can influence: (i) its permeability characteristics and, (ii) modulate enzymatic activities (477). Such changes will have immediate and dramatic effects on cardiac function, viability, and integrity. Protein changes are most obvious as alterations in enzyme activity. However,

gross qualitative and quantitative changes in protein composition were analyzed by protein separation with SDS polyacrylamide gel electrophoresis (122). No major changes in protein composition of sarcolemmal membrane were observed as a fraction of the diabetic state, although a significant increase in protein of approximately 70,000 daltons was identified (122). Major alterations in the lipid composition of sarcolemma isolated from hearts of diabetic rats have been demonstrated (122,158,457). In diabetes, the phospholipid composition of the sarcolemma was dramatically changed. Phosphatidylethanolamine and diphosphatidylglycerol concentrations were depressed whereas lysophosphatidylcholine levels were increased significantly in diabetic preparations in comparison to control values. Other phospholipids were unaltered. These changes in phospholipid composition of the cardiac sarcolemma are thought to be important to cellular function and integrity during diabetes. Lysophosphatidylcholine accumulation in the heart has been associated with electrophysiological abnormalities (478) and permeability changes (479). Therefore, the increase in lysophosphatidylcholine concentration in diabetic sarcolemma may perhaps adequately explain the alterations in electrical activity of the diabetic heart (332,480,481) and evidence of an increase in sarcolemmal membrane leakiness in diabetic hearts (332,480,481).

Phosphatidylethanolamine degradation has also been shown to be associated with defects in sarcolemmal permeability in the myocardium (482). It is thus possible, that the increase in lysophosphatidylethanolamine and the decrease in phosphatidylethanolamine could be important factors in an altering the sarcolemmal membrane permeability in the heart from diabetic animals. In addition, an

increased level of lysophosphatidylchone has been shown to inhibit Na^+-K^+ ATPase activity (483), and therefore, could be causally related to the depression in Na^+-K^+ ATPase (158) and Na^+-pump activities (324) reported for the diabetic cardiomyopathy. Also, lysophosphatidylcholine has been shown to inhibit Na^+-Ca^{2+} exchange activity; this suggests that the accumulation of lipid metabolites may produce heart dysfunction by modifying Ca^{2+} movements across the sarcolemmal membrane (484). The change in diphosphatidylglycerol content may have further relevance to the decrease in sarcolemmal Ca^{2+} binding in the diabetic preparations (122). A recent study has examined the molecular species composition of heart sarcolemmal phosphatidylcholine in diabetic cardiomyopathy (485). These investigators reported that phosphatidylcholine from STZ-diabetic rat heart sarcolemma was highly enriched with 18:0/22:6 and 16:0/22:16 species. Insulin normalized these values. Minor changes in arachidonic acid-containing phosphatidylcholine were also observed. It was suggested that the observed changes in the molecular species composition of diabetic sarcolemma phosphatidylcholine may originate abnormal diacylglycerol species. Their observations further indicated that there may well be alterations in the PKC-dependent phosphorylation processes in the diabetic heart.

The phospholipid N-methylation has been suggested to be important in changing several membrane-associated functions (486) including the control of Ca^{2+} fluxes in the myocardium (487-489). Phosphatidylethanolamine N-methylation has been shown to increase Ca^{2+}-pump activities in heart sarcolemma and SR (488,489). Studies with 6-week STZ-treated rats have shown that phosphatidylethanolamine N-methylation was defective in cardiac sarcolemma membranes (490,491) and

altered in other subcellular membranes, such as the mitochondria and sarcoplasmic reticulum (491). These alterations were reversed with insulin treatment. Cholesterol composition of the sarcolemma isolated from hearts of diabetic rats was also found to be altered (122,158,457). In these studies, total cholesterol content have been shown to increase in diabetic preparations. This is likely to be due to the elevation in plasma cholesterol concentration in these diabetic animals (122,158,457). Alterations in membrane cholesterol content are known to have dramatic effects on enzyme activities (477). Therefore, elevated cholesterol levels in sarcolemma from diabetic animals may induce changes in the sarcolemmal enzyme activities.

Carbohydrate residues associated with the basement membrane component of the cardiac sarcolemmal membrane from diabetic rats were found to be altered. Specifically, the sialic acid residues acid residues in the sarcolemma were significantly reduced in content during diabetes (122). This change in membrane composition could be normalized if diabetic animals were made euglycemic with chronic insulin administration (122). Removal of sialic acid residues from the sarcolemmal membrane has been closely associated with an increase in membrane permeability (492,493). Thus, it is possible, that a decrease in sialic acid content in cardiac sarcolemma from diabetic rats may alter permeability characteristics; this would correlate well with the in vivo evidence of change in integrity of hearts from diabetic animals (239). A "leaky" plasma membrane may also explain reports of altered cation contents (252,494) in myocardium from diabetic animals. The depression in sarcolemmal sialic acid content can also relate to an observed decrease in passive Ca^{2+} binding to this membrane during diabetes

(122). Sialic acid residues represent a source of fixed net negative charge on the external surface of the sarcolemma. Because of this, they are capable of attracting positively charged Ca^{2+} ions. The depression in sialic acid content, therefore, may be associated with the decrease in Ca^{2+} binding to the sarcolemma. Studies using agents which cleaved sialic acid residues from the sarcolemma supported the contention that these residues may play an important role in the defect in passive Ca^{2+} binding (122). It is, however, important to note that conflicting data exist regarding the importance of sialic acid residues in sarcolemmal Ca^{2+} binding (495) and it appears that the alteration in glycoprotein residues may not be the only factor responsible for the defect in membrane Ca^{2+} binding. In this regard, it should be pointed out that acidic phospholipids are capable of binding large quantities of Ca^{2+} (495). Therefore, the decrease in content of the acidic phospholipid, diphosphatidylglycerol, may also be partially responsible for the depression in Ca^{2+} binding to cardiac sarcolemma isolated from diabetic rats (122).

Diabetic animals (STZ/alloxan) and diabetic patients have both been shown to exhibit various kinds of electrophysiological aberrations ranging from an altered action potential, EKG changes, increased arrhythmias and conduction defects. Fein et al (332) reported prolongation of the action potential duration (APD) as well as reduction in the Vmax, resting membrane potential (RMP) and action potential amplitude (APA) in the ventricular muscle from diabetic rats. These changes were suggestive of altered function of sarcolemmal ionic channels. Because calcium ions play an important and decisive role in activating the contraction of different muscles, it had been assumed

that any diabetes-related change of heart contractions may be originated from disturbances in the myocardial calcium homeostasis. Measurements using calcium-sensitive electrodes (496) indicated an approximately 3-times higher resting myoplasmic Ca^{2+} concentration in the left ventricular myocardium of diabetic rats, indicating a disorder in the regulation of the intracellular Ca^{2+} level. The same conclusion was drawn by Allo et al (497) on the basis of fura-2 measurements carried out on cardiomyocyte suspension. Other data, however, suggested a decreased calcium content of isolated diabetic myocytes (498).

Several studies (499-503) by using rat papillary muscles, left ventricular trabeculae and isolated ventricular myocytes have confirmed the observation of Fein et al (332) that a prolongation of the action potential exists in alloxan- and STZ-induced diabetic rats. In addition, delayed after polarizations and triggered activity were observed more frequently in myocardium from diabetic rats than control rats (480). These differences in action potentials as well as the latter observed phenomena are a reflection of alterations in ionic flux in the myocardial cell (480,499-503) or compositional changes in the heart (241). Shimoni et al (503) recently reported that substantial changes, for instance, in the Ca^{2+}-independent transient outward K^+ current (I_t) and the steady-state outward K^+ current (I_K) can be detected in rat ventricular myocytes as early as 4-6 days after STZ-administration. In addition to changes in the action potential and in ion channels, diabetics exhibit alterations in EKG. These alterations have been reported in both insulin- and non-insulin-dependent diabetic patients in the absence of major vessel disease (204,306). The EKG parameters which have been shown to be altered in diabetics include: P-wave charges (505),

prolonged Q-T intervals (279,280,506), increased frequency of QRS abnormalities (294), ST-segment abnormalities (279,294,306,505,506), T-wave charges (279,280,294,306,506) and U-wave alterations (505). Also, a leftward frontal QRS axis (279) and lower EKG voltages (280) have been detected in diabetics. These alterations in electrophysiological properties of diabetic heart are consistent with membrane abnormalities in this disease process.

III. METHODS

A. Experimental Models

1. Alloxan-diabetic rats

Male Sprague-Dawley rats weighing between 200 and 250 g were made diabetic with a single injection of alloxan monohydrate (Sigma), at a dose of 65 mg/kg into the tail vein. This dose yields 100% diabetogenesis. Rats were anesthetized with Halothane 2% and the alloxan was dissolved in a 0.05 M citrate-buffer (pH 4.5) saline solution just prior to injection. Because alloxan has been reported to be capable of nonspecific kidney damage (112,210), five to ten ml of 0.9% NaCl were given intraperitoneally immediately following alloxan administration, as suggested by Heimberg et al (507,508) in order to diminish kidney damage. Since rats given alloxan undergo a transient and dangerously fatal hypoglycemic period, a 50% dextrose-saline solution was administered subcutaneously within 12 - 24 hours after alloxan administration to minimize and prevent mortality. These precautions resulted in a mortality rate of generally less than 2% in this experimental model. Three days after alloxan-injection, rats displaying glycosuria (> 2%) and elevated plasma glucose (> 300 mg/100 ml) were used as the diabetic group. Age-matched control rats were also used, and they received an injection of only the citrate-buffered saline solution, without containing alloxan. Throughout the course of the experimental period, food and water were provided ad libitum. Diabetic rats and age-matched controls were used for hemodynamic, subcellular and molecular studied at the following time points 48 hours (equilibrium period) after alloxan administration: "7-days", "10-days", "2 weeks", "4 weeks", "8 weeks", "12 weeks".

Four weeks after alloxan injection, a random group of diabetic animals were given daily subcutaneous injections of Ultralente Insulin for a period of 4 weeks. The dose of insulin was adjusted to achieve blood glucose levels in the range of 100 - 200 mg/100 ml. The approximate dose of insulin used was 3 U/day. All diabetic rats, insulin-treated diabetic rats and the age-matched control rats were killed at the above mentioned time points by decapitation. Trunk blood was collected at time of death in heparinized tubes and plasma was prepared from the blood samples upon centrifugation at 3000 x g. Plasma was stored at -20°C for the analysis of glucose (Sigma Glucose Reagent Kit) and for RIA analysis of insulin (Linco Rat-Insulin RIA Kit). Hearts were immediately removed and the ventricular tissue was isolated from atria, connective tissue, as well as major blood vessels, weighed and then processed for the isolation of the various subcellular organelles, such as: myofibrils, sarcoplasmic reticulum, and sarcolemma. Ventricular tissue used for subsequent molecular biology studies, were washed and rinsed in a 10 mM EDTA/10 mM solution to remove any adhering blood. These hearts were then placed and stored in liquid nitrogen for subsequent RNA extraction.

2. Alloxan-diabetic rabbits

A second model of diabetes, the alloxan-induced diabetic rabbit was chosen to study subcellular biochemical changes. This species is more prone to atherosclerosis than the rat and can thus allow one to study the influence of atherosclerosis on diabetic cardiomyopathy. In this study, adult male New England white rabbits (1.7 - 2.4 kg) were fasted overnight before injection with alloxan. Alloxan monohydrate was dissolved in sterile NaCl (0.9%) immediately before use and injected (125 mg/kg) into

the caudal ear vein of lightly anaesthetized rabbits (sodium pentobarbital 20 mg/kg, intravenously). Because alloxan injection is irritating and painful, 0.5 ml of 2% xylocaine was injected subcutaneously into the ear just before the injection of alloxan. Since alloxan is capable of producing fatal hypoglycemia as the result of massive insulin release from the pancreas, animals were treated with a 20% glucose (15 -20 ml) subcutaneously every 4 - 6 hr for the first 24 hr following alloxan administration. To prevent dehydration from severe polyuria, intravenous normal saline (0.9 %; 10 ml/kg) was also administered. All rabbits were housed individually and given free access to food and water. During the 10-week period, the diabetic state of the animals was monitored by periodic tests for urine glucose and urine ketones with the use of urine glucose/ketone test paper. In addition, blood glucose levels were monitored (as described for the rat) and plasma insulin levels were determined with the use of RIA kit (Amersham Corp.). Total cholesterol was also determined with a cholesterol kit (Sigma). After 10 weeks, both control and diabetic rabbits were anesthetized with intravenous injection of sodium pentobarbital and hearts were quickly removed and processed for sarcolemma, myofibrils and sarcoplasmic reticulum. Blood was also collected and processed as described for the rat.

B. Hemodynamic Studies on Rats

The animals were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg Nembutal). To maintain adequate ventilation, the trachea was intubated, the right carotid artery was exposed, and a microtip pressure transducer (model SPR-249, Millar) was introduced through a proximal arteriotomy (509,510). The catheter was advanced

carefully through the lumen of the carotid artery until the tip of the transducer entered the left ventricle. The catheter was secured with a silk ligature around the artery and readings were taken from a dynograph recorder (model R5HA, Beckman; Fullerton, CA). Left ventricular systolic pressure (LVSP), rate of contraction (+ dP/dt), rate of relaxation (- dP/dt), heart rate (HR) and left ventricular end-diastolic pressure (LVEDP) were recorded after 10 - 15 min of stabilization.

C. Isolation of Subcellular Fractions

1. Isolation and characterization of myofibrils

The myofibrils were prepared according to the procedure of Solaro et al (511), as described previously (356,357). Briefly, ventricular tissue was homogenized in a Waring blender in 4.0 vol of a medium containing 0.3 M sucrose and 10 mM imidazole (pH 7.0), and the resultant slurry was centrifuged at 17,300 g for 20 min. The particulate fraction was suspended in (mM) 60 KCl, 2 $MgCl_2$, and 30 imidazole (pH 7.0) (Buffer #1) and centrifuged at 750 g for 15 min. The pellet was washed four more times using this procedure until it was finally suspended in Buffer #1 plus 2 mM EGTA and centrifuged again at 750 g. This pellet was washed twice in a medium containing Buffer #1 plus 0.1% Triton X-100. After recentrifugation at 750 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). This suspension was used for different assays within the next 8 hr of isolation. The myofibrillar fraction was found to be devoid of membrane contamination because it showed detectable Na^+-K^+ ATPase, sodium azide sensitive ATPase, and oxalate supported Ca^{2+} uptake activity as determined by methods outlined elsewhere (123,158,394). Protein determination was carried out using bovine serum

albumin as a standard (355).

2. Isolation of sarcoplasmic reticulum

Membrane fraction enriched with SR (microsomal fraction) was isolated according to the method of Harigaya and Schwartz (512) as described elsewhere (123,357). Briefly, the ventricular tissue was homogenized in a Waring blender at medium speed for 45 sec in a medium containing (in mM) 10 $NaHCO_3$, 5 NaN_3, and 15 Tris-HCl (pH 6.8). The homogenate was centrifuged at 10,000 for 20 min, and the supernatant was again centrifuged at 40,000 g. The 10,000 - 40,000 g sediment was suspended in 0.6 M KCl and 20 mM Tris-HCl (pH 6.8) to solubilize contractile proteins and then centrifuged at 40,000 g for 45 min. The final pellet was washed and suspended in 0.25 M sucrose and 20 mM Tris-HCl (pH 6.8).

3. Isolation of sarcolemmal membranes

Purified light sarcolemmal membrane fraction was isolated from ventricular tissue according to the method of Pitts (513). The ventricles were washed, minced, and then homogenized in 0.6 M sucrose, 10 mM imidazole-HCl, pH 7.0 (3.5 ml/g tissue) with polytron PT-20 (5 x 20 s, setting 5). The resulting homogenate was centrifuged at 12,000 g for 30 min, and the pellet was discarded. After diluting (5 ml/g tissue) with 140 mM KCl - 20 mM 3-(N-morpholino)-propanesulphonic acid (MOPS), pH 7.4 (at 37°C, the supernatant was centrifuged at 95,000 g for 60 min. The resultant pellet was suspended in 140 mM KCl, 20 mM MOPS, pH 7.4 buffer and layered over a 30% sucrose solution containing 0.3 M KCl - 50 mM $Na_4P_2O_7$, and 0.1 M Tris (hydroxymethyl) aminomethane; (Tris)-HCl, pH 8.3. After centrifugation at 95,000 g for 90 min (using a Beckman swinging bucket motor) the band at the sucrose-buffer interface was

taken and diluted with 3 vol of 140 mM KCl, 20 mM MOPS, pH 7.4 (at 37°C). A final centrifugation at 95,000 g for 30 min resulted in a pellet rich in sarcolemma. All isolation steps, as for myofibrils and SR, were carried out at 0 - 4°C. The final pellet was suspended in 0.25 M sucrose - 10 mM histidine, pH 7.2 (3.5 mg/ml) and then quickly frozen in liquid N_2. Protein concentrations of all membrane was determined by the method of Lowry et al (514).

4. Myosin isozymes composition

After decapitation and the removal of hearts from the rats, 10 - 20 mg portions of left ventricle were frozen in liquid N_2 for determination of myosin isoenzymes. Myosin isoenzymes were separated by electrophoresis of native proteins in non-dissociating medium containing 20 mM $Na_4P_2O_7$ according to the method of Hoh et al (515) with slight modifications. The contractile proteins were electrophoresed in a 1.5 mm thick and 12 cm long slab gel of 4% total, 3% cross-linkage, 10% glycerol, and 20 mM $Na_4P_2O_7$ (pH 8.8), using a Pharmacia GE 214 apparatus, at 0 - 5°C. The electrophoresed buffer (20 mM $Na_4P_2O_7$ and 1% glycerol, pH 8.8) was recirculated between the lower and upper chamber. The running buffer was cooled to 0 - 2°C, using immersed cooling coils of the apparatus. The gels were pre-run for 45 min at 120 V (constant), and then 5 - 15 ug protein was electrophoresed for 18 - 20 hr at 120 V. Gels were stained in 0.25% Coomassie blue R250 for 2 hr and destained by diffusion in 7% acetic acid for several hours. These electrophoresed gels were scanned on a laser densitometer, and the percentage of different isoforms of myosin was calculated.

D. Subcellular ATPase and Calcium Uptake Assays

1. Myofibrillar ATPase activity

For the myofibrillar ATPase assays, the solution contained (in mM): 52.6 KCl, 25 imidazole, 3 $MgCl_2$, 5 NaN_3, 1 EGTA. Free calcium concentrations of the medium ranged from 0.1 to 10 uM. The Ca^{2+}-stimulated ATPase activity of myofibrils was measured at 30°C in the above medium (pH=7.0) containing $1x10^{-5}$ M free Ca^{2+} at different incubation times. The reaction was started with 300-500 ug/ml of protein and stopped with 1 ml of 12% ice-cold tricholoroacetic acid (TCA). The samples were centrifugued and inorganic phosphate in protein free supernatant was determined as indicated previously (356).

2. SR Ca^{2+} uptake and ATPase activities

Ca^{2+} uptake activities of SR were determined using the Millipore filtration technique. The membranes (0.03-0.07 mg/ml) were pre-incubated at37°C (total volume 1 ml) in (in mM) 100 KCl, 20 Tris·HCl (pH 6.8), 5 $MgCl_2$, 5NaN_3, 5 K-oxalate, and 1 EGTA as well as 10 uM free Ca^{2+} (123). The reaction was started by the addition of 5 mM Tris·ATP and was terminated at desired times by filtering 100 ul aliquots of the incubation mixture through Millipore filters (0.45 um pore size). The filters were washed with 5 ml of 20 mM Tris/100 mM HCl (pH 7.4), dried overnight, and then counted for radioactivity by using the standard liquid scintillation counting technique. Appropriate blanks in the absence of ATP were subtracted to calculate the ATP-dependent Ca^{2+} uptake. Total (Mg^{2+} + Ca^{2+}) and basal (Mg^{2+}) ATPase were determined in an incubation medium similar to that used for the $^{45}Ca^{2+}$ uptake assay, except that when total ATPase was measured, nonradioactive $CaCl_2$ (final 10 uM free Ca^{2+}) was used and when basal ATPase was measured, Ca^{2+} was omitted and 0.2 mM EGTA was added. The reaction was started by the addition of 5 mM Tris-ATP after a 3-min

preincubation period in presence of 30-60 ug of membrane protein and terminated after 5 min by 1 ml of 12% ice-cold TCA. Inorganic phosphate liberated in the protein-free filtrate was assayed as previously described (356). The Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activity reported here is the difference between the total and basal ATPase activities.

3. Sarcolemma

a. Na^{+}-dependent Ca^{2+} uptake

Na^{+}-dependent Ca^{2+} uptake measurement was carried out by a method described in detail elsewhere (516). In short, 5 ul of sarcolemmal vesicles (1.5 mg/ml; 7.5 ug protein/tube) preloaded with NaCl/MOPS buffer at 37°C for 30 min were rapidly diluted 50 times with Ca^{2+} uptake medium containing 140 mM KCl, 20 mM MOPS, 0.4 uM valinomycin, 0.3 uCi $^{45}Ca^{2+}$ and various Ca^{2+} concentrations (5-80 uM), pH 7.4 (at 37°C). After the appropriate time span, the reaction was stopped by the addition of ice-cold 0.03 ml solution containing 140 mM KCl, 1 mM $LaCl_3$, 20 mM MOPS, pH 7.4 (at 37°C). Samples (0.25 ml from 0.28 ml of the total reaction mixture) were filterd through Millipore filters (pore size=0.45 um) and washed twice with 2.5 ml of ice-cold 0.03 ml stopping solution containing 140 mM KCl, 0.1 mM $LaCl_3$, 20 mM MOPS, ph 7.4, at 37°C. Radioactivity of filters were measured using a Bckman LS 1701 Counter. In parallel to these samples, nonspecific Ca^{2+} uptake was measured by placing the Na^{+}-loaded sarcolemmal vesicles in Ca^{2+} uptake medium which contained 140 mM NaCl instead of KCl. Na^{+}-dependent Ca^{2+} uptake activity was corrected by subtraction of the non-specific Ca^{2+} uptake values.

b. Sarcolemma calcium pump activity

For the determination of Mg^{2+} ATPase and Ca^{2+} stimulated ATPase activities, experimental conditions were the same as reported elsewhere (517,518) with some modificiations. Sarcolemmal vesicles (resuspended in 140 mM KCl, 20 mM MOPS, pH 7.4 at 37°C; 25 ug protein/tube) were preincubated at 37°C for 5 min in 0.5 ml of medium containing 140 mM KCl, 20 mM MOPS, pH 7.4 at 37°C, 2 mM $MgCl_2$, 5 mM NaN_3, 0.1 mM EGTA, 2.5 mM phosphoenolpyruvate (PEP), and 10 I.U./ml puruvate kinase. The reaction for Mg^{2+} ATPase was started by the addition of 4 mM Tris ATP, pH 7.4 at 37°C, and terminated 5 min later with 0.5 ml of 12% ice-cold TCA. The liberated phosphate was measured as before (356). Estimation of total (Ca^{2+} + Mg^{2+})-ATPase was made in the above mentioned medium containing $5x10^{-6}$ M free Ca^{2+} instead of EGTA. Mg^{2+} ATPase and free Ca^{2+} concentration in the incubation medium was calclulated (as done for other assays) using the "SPECS" FORTRAN program of Fabiato (519). The Ca^{2+}-stimulated ATPase activity was the difference between the total ATPase and the Mg^{2+}-ATPase activities.

In order to measure ATP-dependent Ca^{2+} accumulation ("uptake") (517), sarcolemmal vesicles (22.5 ug protein/tube) were preincubated at 37°C for various times in 0.20 ml of medium containing 140 mM KCl, 10 mM MOPS, pH 7.4, at 37°C, 2 mM $MgCl_2$, and $^{45}CaCl_2$-EGTA (which contained $5x10^{-6}$ M free Ca^{2+}). Ca^{2+} accumulation was initiated by the addition of 4 mM Tris-ATP, pH 7.4 (at 37°C). 180 ul aliquots were immediately filtered through Millipore filters (pore size=0.45 uM), washed twice with 2.5 ml ice-cold 140 mM KCl, 20 mM MOPS, and 0.1 mM $LaCl_3$, pH 7.4 (at 37°C), dried, and radioactivity determined for calculating the total Ca^{2+} accumulation. Nonspecific Ca^{2+} binding was measured in the absence of ATP for each set of experiments and the

ATP-dependent Ca^{2+} accumulation (uptake) was calculated by subtracting nonspecific Ca^{2+} binding from the total Ca^{2+} accumulation.

c. Na^+-K^+ ATPase

Estimation of Na^+-K^+ ATPase activity was carried out by a previously described method (520) with some modifications. Briefly, sarcolemmal vesicles (10 ug) were preincubated at 37°C, 1.0 mM EGTA (Tris), pH 7.4 at 37°C, 50 mM histidine-HCl, pH 7.4 at 37°C, 5 mM NaN_3, 2.5 mM PEP, 100 mM NaCl, 10 mM HCl, 6 mM $MgCl_2$, and 10 I.U./ml pyruvate kinase. The reaction was started by the addition of 0.025 ml 80 mM Na_2 ATP, pH 7.4, and terminated after 10 min with 0.5 ml ice-cold 12% TCA. The liberated phosphate was measured as before, by the method of Jaussky and Shorr (521). In some experiments, 1 mM ouabain was added to the reaction medium before the addition of the sarcolemmal preparation. In parallel experiments, either Na^+ plus K^+ or Mg^{2+} was omitted from the incubation medium. Na^+-K^+ ATPase activity was calculated as the difference between activities with and without Na^+ plus K^+. Mg^{2+} ATPase activity was estimated as the difference between the activities registered with and without Mg^{2+} in the absence of Na^+ and K^+ in the medium. All measurements were carried out in duplicate. The concentrations of Mg ATP were calculated using the "SPECS" FORTRAN program developed by Fabiato (519).

E. Molecular Studies

1. RNA preparation

Total RNA from frozen left ventricular tissue samples was prepared according to the protocol of Chomczinski and Sacchi (522), using the guanidinium thiocyanate-phenol-chloroform extraction procedure. Total RNA was dissolved in suitable small volumes of millipore water. The

amount of RNA present was determined by UV absorption.

2. Northern analysis and DNA probes

Denatured RNA samples were electrophoresed on 1.2% agarose gels according to the methods outlined by Sambrook et al (523). Transfer of RNA was accomplished on NT equivalent and positively charged nylon using zeta-probe GT paper. Methods were followed according to the manufacturer's instruction manual (section 2.2 - Northern Blotting). Transfer of RNA onto nylon occurred by capillary transfer for 20 hours. Following this, RNA was fixed via alkaline fixation at 1 hour at 80°C. RNA immobolized on nylon was hybridized with various probes for SR Ca^{2+}-ATPase (SERCA2), alpha-myosin heavy chain (alpha-MHC) as well as sarcolemmal Na^{+}-Ca^{2+} exchange and the $alpha_1$-subunit of the Na^{+}-K^{+} ATPase protein. Scanning densitometry was performed on autoradiograms to estimate the abundance of each particular mRNA species in control, diabetic and insulin-treated rats. Corresponding GAPDH densities were used to normalize all the density values for the above mentioned mRNA transcripts. The GAPDH cDNA was a full length Klenow fragment cDNA (human). The cDNA probe was labelled using a random priming labelling kit purchased through GIBCO. The clone, pHcGAPNR hybridizes to a 1.3 kb band.

The alpha-MHC was obtained from a rat cDNA clone. The 3'-untranslated region was 5'-GGG ATA GCA ACA GCG AGG CTC TTT CTG CTG GAC AGG TTA-3'. This information was kindly supplied by Dr. C.C. Liew (Toronto, ON). This oligo probe was synthesized by the use of a T_4 5'-end polynucleotide kinase (PNK, Boeringer Mannheim) in the laboratory of Dr. I.M.C. Dixon (St. Boniface General Hospital Research Centre, Winnipeg, MB, CAN) using a Beckman oligo synthesizer (Beckman Oligo 1000

DNA Synthesizer). The $alpha_1$-Na^+-K^+ ATPase subunit probe used was the rat 5'-specific $alpha_1$ isoform, which was a 332 bp fragment. This was obtained from Dr. Jerry Lingrel (University of Cincinnati, Cincinnati, OH). This fragment hybridizes to a 3.7 Kb mRNA transcript. The Na^+-Ca^{2+} exchange transcript was hybridized with a cDNA probe obtained from a pA4E2 canine clone (obtained from Dr. Ken Philipson, Los Angeles, CA) of the Na Ca exchanger. The pA4E2 is a 1 Kb insert of the 3'-transcribed end. This probe cross reacts well with other species. This probe hybridizes with a 7 kb on Northern blots. The SERCA2, RSB87 plasmid (obtained from A.K. Grover) used the probe bound to a 4.6 kb position on Northern blots.

F. Statistical Analysis

Results are expressed as means ± SE. Statistical analysis was carried out by Student's t-test, and a P level < 0.05 was taken to reflect a significant difference between control and experimental

IV. RESULTS

A. General Characteristics and Hemodynamic Changes in Experimental Rats

The data in Table 1 indicate that the diabetic rats had significantly lower body weights (-25%) and ventricular weights (-24%) 1 week after alloxan administration. In addition, 4 weeks after alloxan administration, diabetic animals exhibited a significantly higher heart-to-body weight ratio. In these experimental animals, the presence of diabetes was confirmed by markedly elevated plasma glucose levels (3.4 fold increase) and severely depressed plasma insulin levels (3.5 fold decrease). Daily injections of insulin to the 4 week diabetic rats for 4 weeks fully normalized plasma glucose and insulin levels as well as ventricular and body weights. Assessment of hemodynamic parameters (Table 2) in the diabetic rats compared to the control groups revealed significant alterations in mean arterial pressure, heart rate, left ventricular end-diastolic pressure, left ventricular systolic pressure, rate of contraction (+dP/dt) and rate of relaxation (-dP/dt). Specifically, a pronounced depression (-16%) in the rate of relaxation was observed as early as 2 weeks after alloxan administration; this parameter continued to further decline after this time point where it represented an average 57% depression compared to control value. By 4 weeks after alloxan administration, mean arterial pressure, heart rate, left ventricular systolic pressure and +dP/dt were all reduced significantly. Diabetic rats at this time point exhibited a marked increase in left ventricular end-diastolic pressure. All of these above hemodynamic indices were subsequently normalized upon insulin administration.

B. Myofibrillar ATPase Activities and Myosin Isoenzyme Profile

Table 1: General characteristics of control, diabetic and insulin treated diabetic rats.

Group	Body(B) Weight (g)	Ventricle (V) Weight (mg)	Ventricle: Body Weight Weight (mg/g)	Plasma Glucose (mg/dL)	Plasma Insulin (ng/mL)
1 Week					
Control	250 ± 10	725 ± 42	2.90 ± 0.06	158 ± 15	2.95 ± 0.20
Diabetic	192 ± 7*	555 ± 57*	2.89 ± 0.08	523 ± 28*	0.84 ± 0.17*
10 Days					
Control	268 ± 9	768 ± 30	2.90 ± 0.07	148 ± 19	3.01 ± 0.15
Diabetic	195 ± 8*	559 ± 46*	2.87 ± 0.04	557 ± 31*	0.89 ± 0.15*
2 Weeks					
Control	283 ± 12	821 ± 45	2.91 ± 0.09	152 ± 10	3.00 ± 0.17
Diabetic	202 ± 6*	572 ± 32*	2.84 ± 0.07	525 ± 19*	0.91 ± 0.16*
4 Weeks					
Control	330 ± 22	912 ± 51	2.76 ± 0.08	150 ± 12	3.04 ± 0.11
Diabetic	185 ± 9*	540 ± 27*	3.10 ± 0.05*	495 ± 26*	0.88 ± 0.14*
8 Weeks					
Control	475 ± 20	1170 ± 63	2.46 ± 0.11	154 ± 13	3.10 ± 0.18
Diabetic	225 ± 12*	675 ± 29*	3.00 ± 0.15*	520 ± 24*	0.82 ± 0.20*
Insulin treated	345 ± 25*	895 ± 47	2.59 ± 0.07	162 ± 13	3.21 ± 0.15
12 Weeks					
Control	535 ± 23	1340 ± 56	2.50 ± 0.05	166 ± 19	3.14 ± 0.19
Diabetic	249 ± 13*	749 ± 35*	3.00 ± 0.12*	512 ± 27*	0.93 ± 0.18

Values are means ± SE of 10-15 animals per experimental group. * Significant from Control, $P<0.05$.

Table 2: Hemodynamic parameters of control, diabetic and insulin treated diabetic rats.

Group	HR (Beat/min)	MAP (mmHg)	LVEDP (mmHg)	LVSP (mmHg)	+dP/dt (mmHg/s)	-dP/dt (mmHg/s)
1 Week						
Control	383 ± 19	120 ± 7	2.5 ± 1.2	132 ± 8	5106 ± 223	4703 ± 215
Diabetic	378 ± 15	117 ± 2	3.0 ± 1.1	130 ± 7	4800 ± 79	4450 ± 220
10 Days						
Control	378 ± 22	122 ± 9	2.6 ± 1.0	128 ± 5	5005 ± 159	4801 ± 189
Diabetic	365 ± 17	114 ± 8	3.0 ± 1.2	131 ± 7	4850 ± 207	4587 ± 158
2 Weeks						
Control	384 ± 18	125 ± 9	2.4 ± 1.3	125 ± 7	5017 ± 200	4754 ± 188
Diabetic	368 ± 15	117 ± 5	3.5 ± 1.2	125 ± 8	4750 ± 188	3999 ± 200*
4 Weeks						
Control	388 ± 19	117 ± 5	2.5 ± 1.0	129 ± 8	4951 ± 175	4725 ± 150
Diabetic	313 ± 21*	99 ± 4*	6.2 ± 1.1*	110 ± 5*	3065 ± 212*	2850 ± 161*
8 Weeks						
Control	391 ± 15	123 ± 7	2.4 ± 1.1	129 ± 7	4983 ± 160	4805 ± 122
Diabetic	280 ± 17*	93 ± 5*	6.1 ± 1.0*	104 ± 4*	2841 ± 270*	2150 ± 207*
Insulin treated	385 ± 15	109 ± 10	2.7 ± 1.1	125 ± 4	4895 ± 152	4658 ± 125
12 Weeks						
Control	394 ± 16	130 ± 9	2.0 ± 1.3	128 ± 5	5055 ± 194	4933 ± 170
Diabetic	277 ± 14*	98 ± 7*	6.2 ± 1.4*	107 ± 7*	2707 ± 155*	2107 ± 210*

Values are means ± SE of 6 - 7 experiments. MAP = Mean Arterial Pressure; HR = Heart Rate; LVEDP = Left Ventricular End Diastalic Pressure; LVSP = Left Ventricular Systolic Pressure; dP/dt = Rate of Contraction; -dP/dt = Rate of Relaxation. * Significant from Control, $P<0.05$.

Myofibrils isolated from diabetic animal hearts exhibited a significant depression (-46%) of Ca^{2+}-stimulated ATPase activity and a significant depression of Mg^{2+}-stimulated ATPase activity compared with control hearts, 4 weeks after alloxan administration (Table 3). From Fig. 1 it is evident that myofibrils isolated from diabetic rat hearts showed a significantly lower Ca^{2+}-stimulated ATPase activity than control preparations at all incubation times; insulin administration for 4 weeks significantly improved the ATPase activity in these studies. Figure 2 shows the values of myofibrillar Ca^{2+}-stimulated ATPase activities at different concentrations of free Ca^{2+} (pCa) for control, diabetic and insulin treated diabetic animals. It should be noted that higher levels of free calcium (>pCa=6.25) revealed a marked depression in Ca^{2+}-ATPase activities in diabetic myofibrils; this change was also restored with 4 weeks of insulin administration.

In order to determine whether the observed results were confounded by contamination of the myofibrils with other subcellular membranes, the myofibrillar fraction was characterized with regard to various key marker enzyme activities. Myofibrillar protein yield (mg/g wet weight) for the control, diabetic and insulin treated rats were 42.4±1.2, 39.8±1.3 and 44.0±2.6, respectively and did not differ amongst groups. Myofibrillar ATPase activities were insensitive to 5 mM sodium azide, a response that would suggest an absence of mitochondrial contamination. The myofibrillar fractions from all three groups also demonstrated no detectable oxalate-supported Ca^{2+} uptake activity and ouabain-sensitive ATPase activity, which would indicate that SR and sarcolemmal contamination was also negligible. Overall, these observations would thus suggest that the relative purity of the preparations

Table 3: Myofibrillar Mg^{2+}-ATPase and Ca^{2+}-stimulated ATPase activity in control, diabetic and insulin treated diabetic rats.

Group	Mg^{2+} ATPase Activity (umol Pi/mg/hr)	Ca^{+2} ATPase Activity (umol Pi/mg/hr)
1 Week		
Control	3.90 + 0.33	12.75 + 0.90
Diabetic	3.83 + 0.41	12.05 + 0.85
10 Days		
Control	3.858 + 0.310	12.96 + 0.80
Diabetic	3.805 + 0.401	12.50 + 0.77
2 Weeks		
Control	3.795 ± 0.305	12.88 ± 0.96
Diabetic	3.812 ± 0.400	12.00 ± 0.70
4 Weeks		
Control	3.775 ± 0.395	12.80 ± 0.91
Diabetic	3.800 ± 0.401	6.85 ± 0.74*
8 Weeks		
Control	3.880 ± 0.312	12.07 ± 0.87
Diabetic	3.809 ± 0.357	6.55 ± 0.71
Insulin treated	3.894 ± 0.299	13.10 ± 0.98
12 Weeks		
Control	3.905 ± 0.411	12.95 ± 0.94
Diabetic	3.778 ± 0.333	6.37 ± 0.72

Results are means ± SE of 5 - 8 myofibril preparations for each group. * Significant from Control, $P<0.05$.

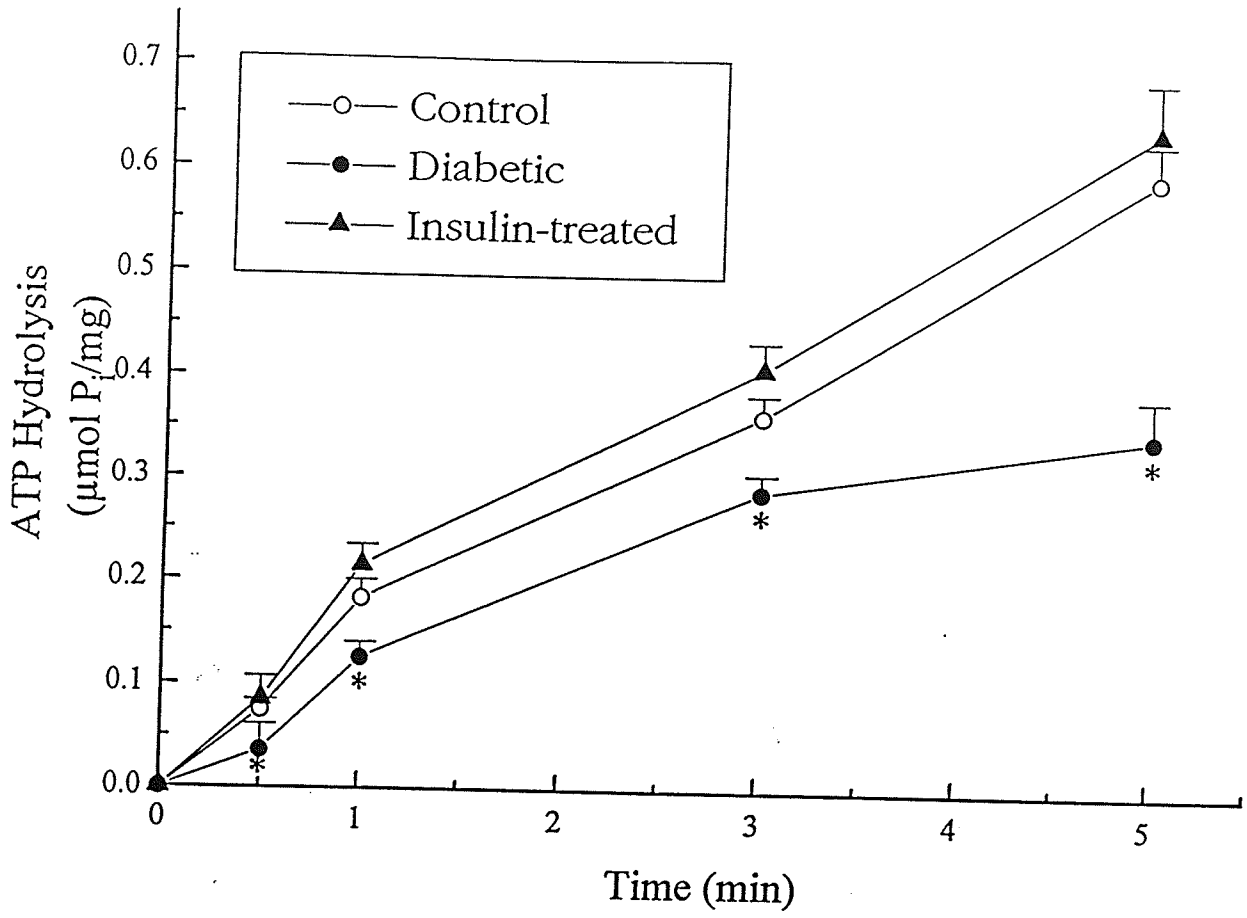

Figure 1. Myofibrillar Ca^{2+}-stimulated ATPase activity examined over various times of incubation in control, diabetic and insulin-treated diabetic rats. Values are means ± SE of 5 to 8 experiments. *Significantly different ($P < 0.05$) from control.

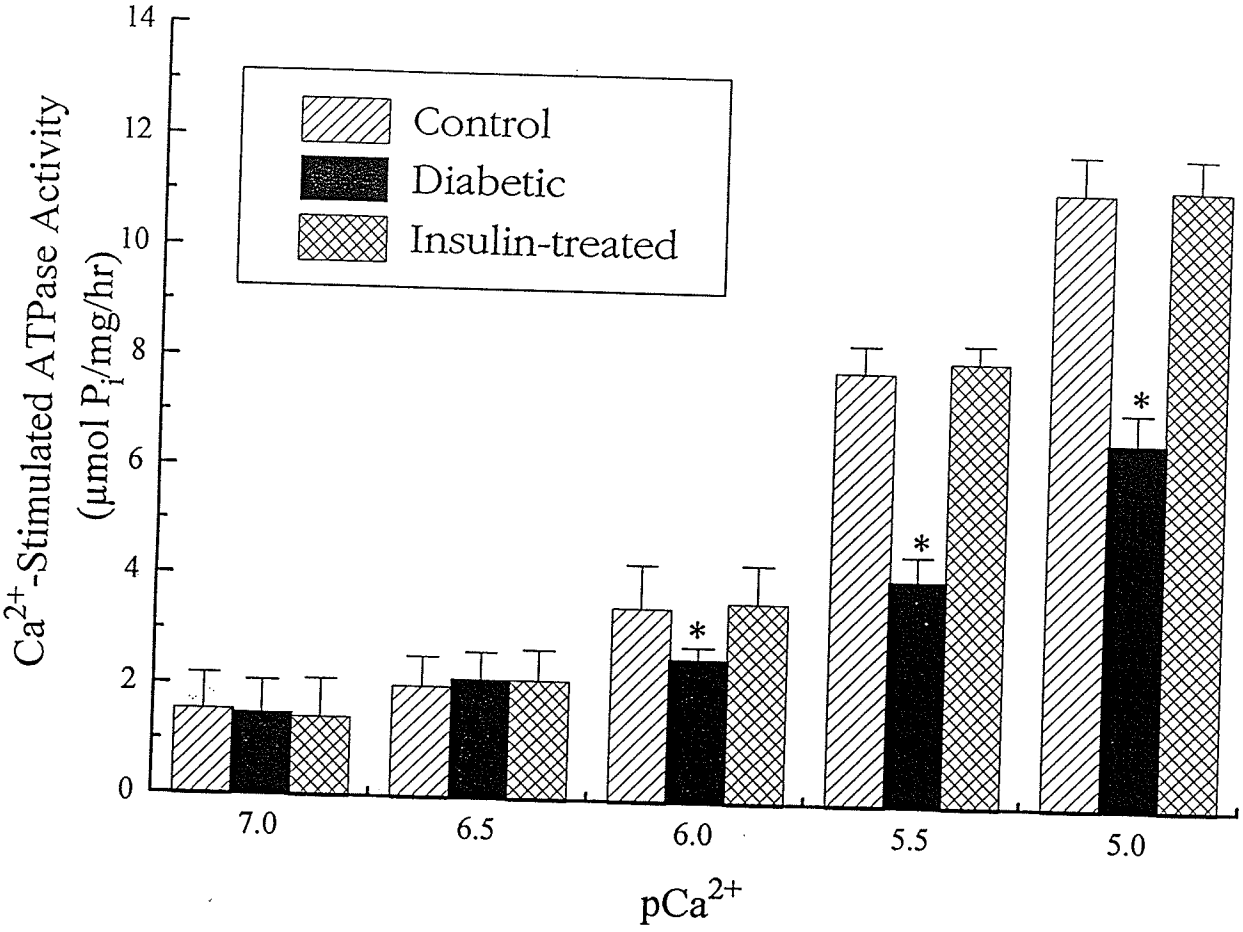

Figure 2. Myofibrillar Ca^{2+}-stimulated ATPase activity at different concentrations of Ca^{2+} in control, diabetic and insulin-treated diabetic rats. Reaction time was 1 min at 30°C. Values are means ± SE of 5 to 8 experiments. *Significantly different ($P < 0.05$) from control.

was apparently similar between all the groups in this study.

An examination of the myosin isoenzyme composition was undertaken in the control, diabetic and insulin treated rats. The results of this study are presented in Fig. 3. Control hearts contained on average 55-65% V_1 myosin, 20-30% V_2 myosin and 10-20% V_3. Diabetic rats after 2 weeks of alloxan administration showed marked concomitant changes in both V_1 and V_3 myosin contents: V_3 averaged 50%, whereas V_1 averaged 18%. V_2 remained relatively stable across all diabetic groups, in the range of 25-30%. Insulin administration to the 4 week diabetic group for a period of 4 weeks normalized the V_1 and V_3 composition.

C. SR Ca^{2+} Transport

ATP-dependent Ca^{2+} uptake activities in SR fractions were examined in control, diabetic and insulin treated diabetic rats. The data in Table 4 indicates that a depression in ATP-dependent Ca^{2+} uptake was depressed by 15% 10 days after alloxan administration. A time-course study of Ca^{2+} uptake (Fig. 4) revealed a marked depression in Ca^{2+} uptake at all the incubation times. After 10 min of incubation, ATP-dependent Ca^{2+} uptake in diabetic SR preparations was only 70% of the control value. Figure 5 shows the values of ATP-dependent Ca^{2+} uptake for control, diabetic, and insulin treated diabetic animals at various free Ca^{2+} (pCa) concentrations. ATP-dependent SR Ca^{2+} uptake was much reduced in diabetics compared to control rats at pCa^{2+} values 6.5 and higher. The V_{max} for the diabetic SR, ATP-dependent Ca^{2+} uptake was 259±7 nmol Ca^{2+}/mg/2 min, whereas that for the Control was 550±12 nmol Ca^{2+}/mg/protein. The dissociation constants (Kd) for SR Ca^{2+} uptake in the diabetic hearts

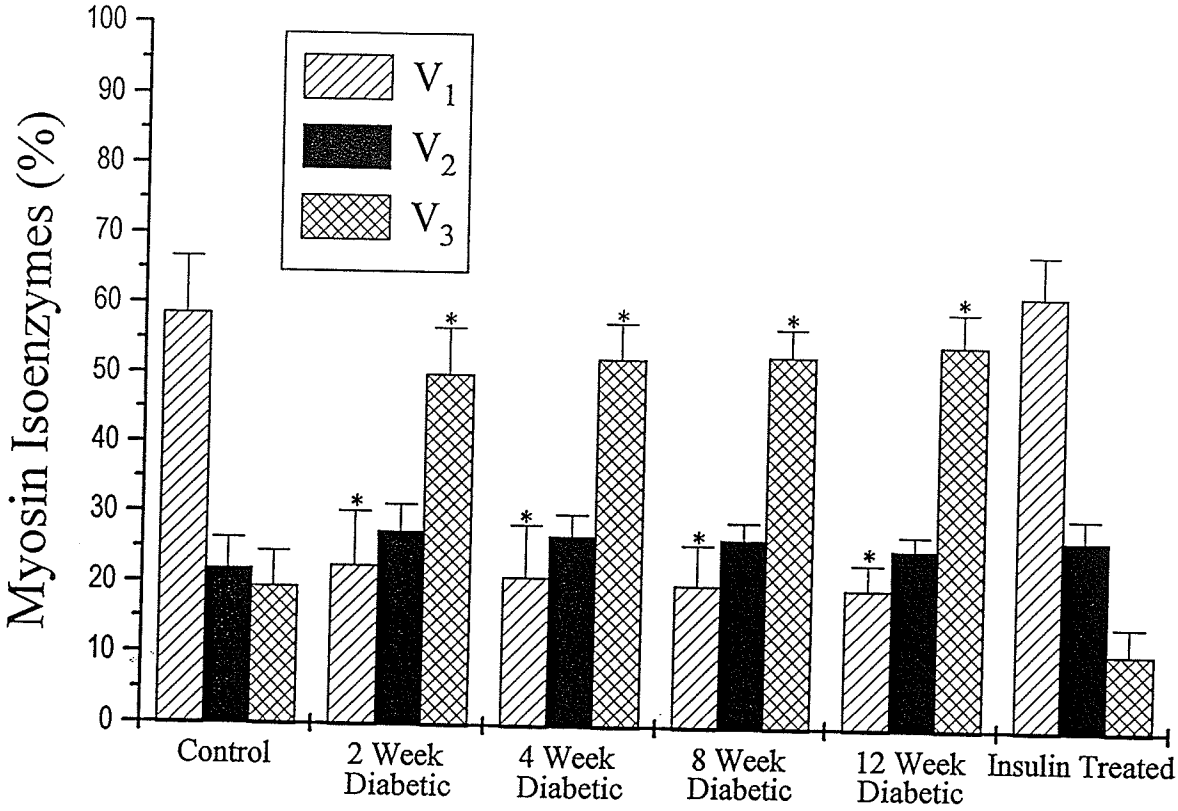

Figure 3. Composition of myosin isoenzymes (V_1, V_2 and V_3) in ventricular tissues from control, diabetic and insulin-treated diabetic rats. Values are means ± SE of 3 to 4 experiments. *Significantly different ($P < 0.05$) from control.

Table 4: Cardiac sarcoplasmic reticulum Ca^{2+} uptake activity in control, diabetic and insulin treated diabetic rats.

Group	Ca^{2+} Uptake (nmol Ca^{2+}/mg protein/min) ATP -dependent	Non - Specific
1 Week		
Control	135 ± 9	15 ± 7
Diabetic	119 ± 15	18 ± 4
10 Days		
Control	127 ± 10	17 ± 5
Diabetic	85 ± 13*	14 ± 8
2 Weeks		
Control	120 ± 8	21 ± 6
Diabetic	65 ± 7*	19 ± 7
4 Weeks		
Control	130 ± 10	20 ± 7
Diabetic	50 ± 12*	19 ± 6
8 Weeks		
Control	133 ± 15	22 ± 8
Diabetic	61 ± 9*	23 ± 5
Insulin Treated	139 ± 12	22 ± 5
12 Weeks		
Control	121 ± 7	18 ± 6
Diabetic	64 ± 11*	21 ± 7

Values are means ± SE of 4 - 6 experiments. * Significant from Control, $P<0.05$. The concentration of free Ca^{2+} was 10um.

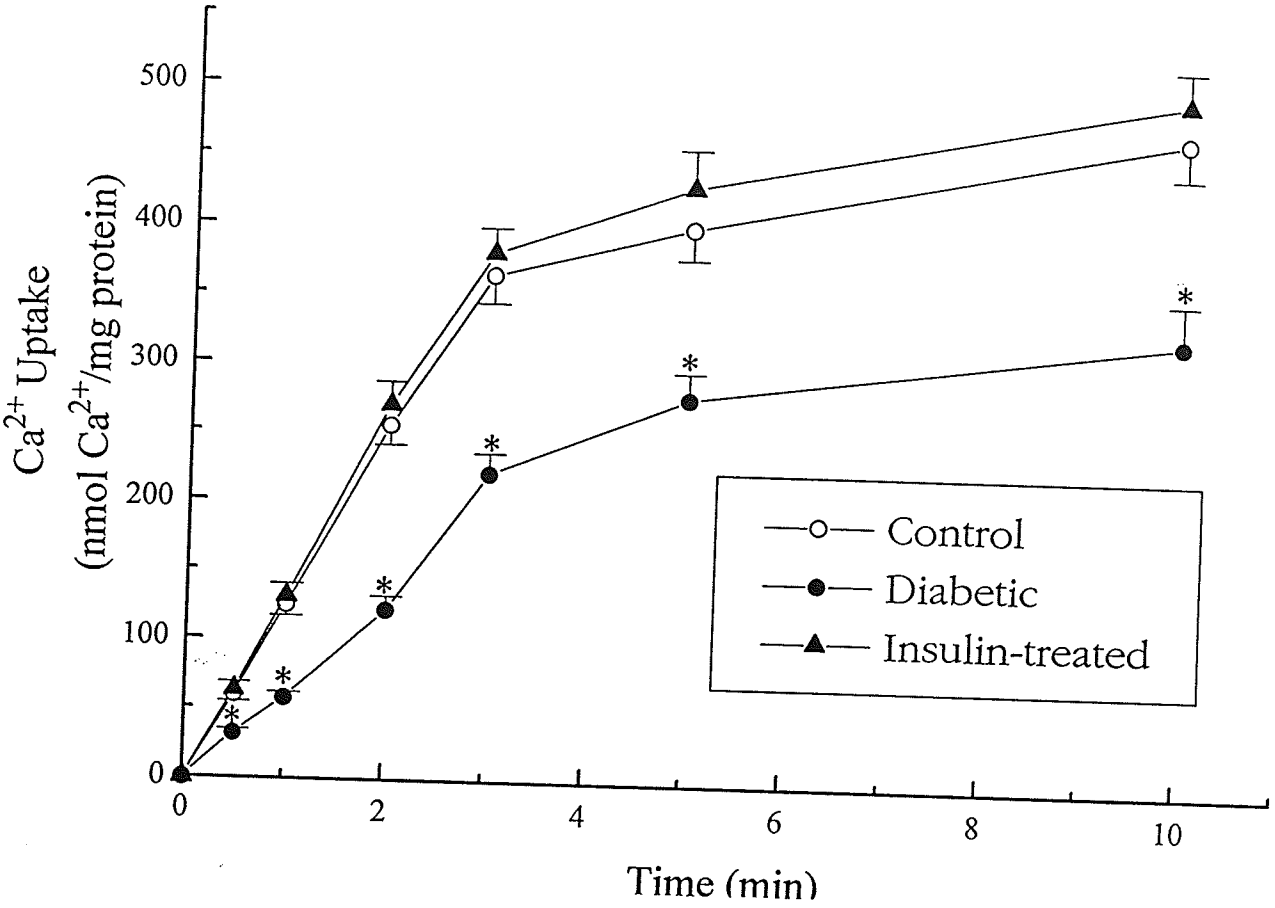

Figure 4. Cardiac sarcoplasmic reticulum Ca^{2+} uptake activity at various times of incubation in control, diabetic and insulin-treated diabetic rats. Values are means ± SE of 5 to 6 experiments. ATP-dependent Ca^{2+} uptake was measured in the presence of 10 uM free Ca^{2+}. *Significantly different ($P < 0.05$) from control.

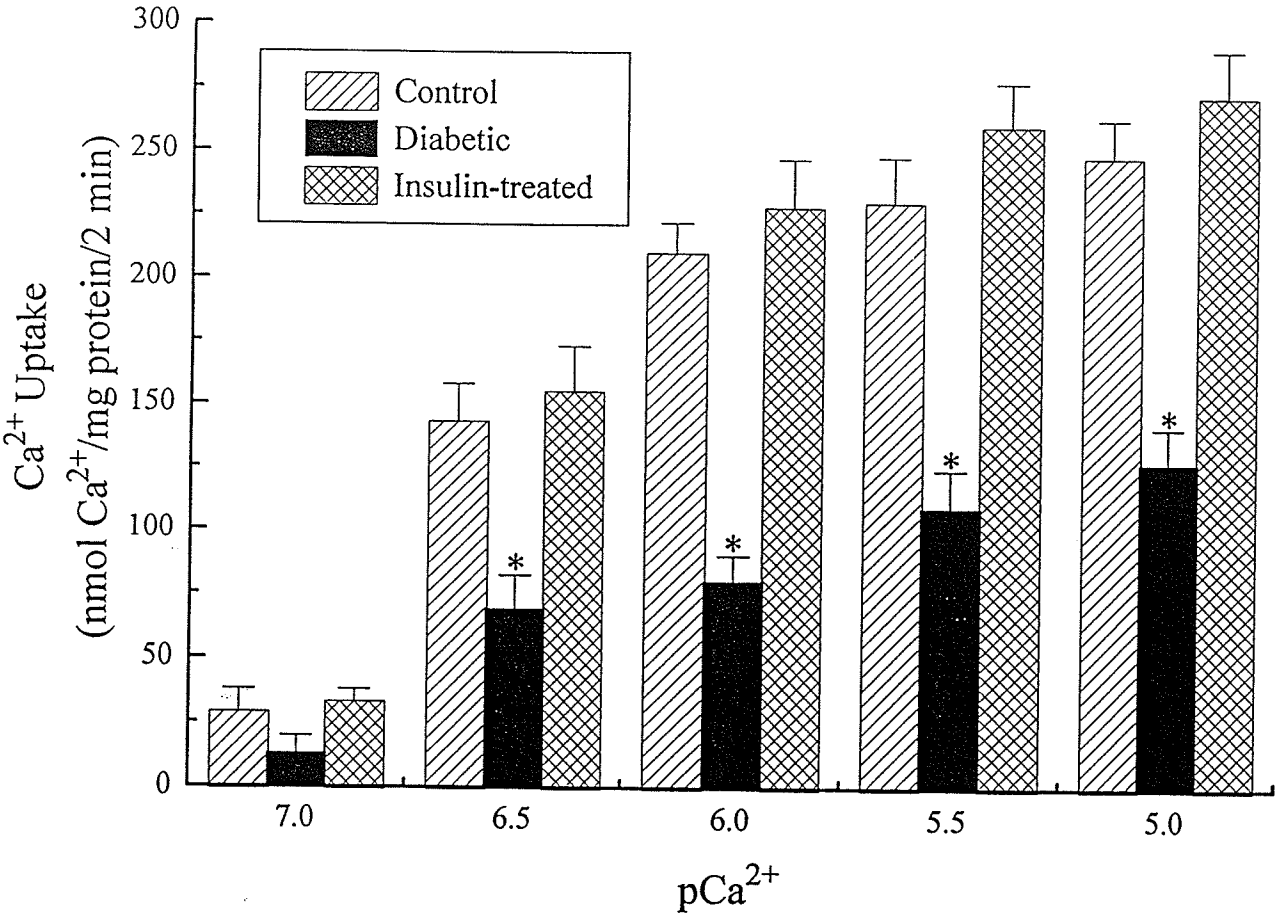

Figure 5. Cardiac sarcoplasmic reticulum Ca^{2+} uptake activity at different concentrations of Ca^{2+} in control, diabetic and insulin-treated diabetic rats. Values are means ± SE of 4 to 6 experiments. *Significantly different ($P < 0.05$) from control.

(Kd=1.811±0.15 uM) and control hearts (Kd=1.734±0.12) were not different. Insulin administration for 4 weeks to the diabetic rats normalized SR ATP-dependent Ca^{2+} uptake (Table 4, Figs. 4 & 5).

SR Ca^{2+}- and Mg^{2+}-stimulated ATPase activities were also examined in control, diabetic and insulin treated rats (Table 7). In diabetic rats compared to controls, significant depressions in both Mg^{2+}-ATPase and Ca^{2+}-stimulated ATPase activities were observed at 7 and 10 days after alloxan administration, respectively. Taking into account all the diabetic groups and control groups, SR Ca^{2+}-stimulated ATPase and SR Mg^{2+}-ATPase declined by 32% and 28% in diabetics in comparison to controls, respectively. Insulin administration for 4 weeks also normalized the SR parameters (Table 7).

Because Ca^{2+} uptake measured by Millipore filtration technique represents the net Ca^{2+} accumulation in SR vesicles as Ca^{2+} influx and Ca^{2+} efflux occur simultaneously during the incubation period, it can be argued that the observed changes in SR Ca^{2+} uptake are due to differences in Ca^{2+} efflux activities in the control, diabetic and insulin-treated diabetic preparations. This possibility was ruled out by including ruthenium red, an inhibitor of SR Ca^{2+} efflux in the incubation medium. The data in Table 5 indicate that the pattern of changes in Ca^{2+} uptake activities in the experimental preparations in the presence of ruthenium red was similar to that seen in its absence (Table 4). Similar changes in the SR Ca^{2+} uptake activities from control, diabetic and insulin treated rat SR preparations were seen when 100-500 uM ryanodine, another inhibitor of Ca^{2+} release, was included in the incubation medium. The absence of 5 mM sodium azide, an inhibitor of mitochondrial Ca^{2+} transport, or the presence of 4 uM vanadate, an

Table 5: Cardiac sarcoplasmic reticulum Ca^{2+} uptake activity in control, diabetic and insulin treated rats in the presence of rthenium red, vanadate, and in the absence (-) of sodium azide.

Group	Ca^{2+} Uptake (nmol Ca^{2+}/mg protein/min)		
	(+) Ruthenium Red	(+)Vanadate	(-) Sodium Azide
Control	135 ± 9	129 ± 6	138 ± 7
Diabetic	68 ± 4*	62 ± 5*	65 ± 4*
Insulin Treated	120 ± 8	122 ± 7	115 ± 10

Values are means ± SE of 3 experiments for each group. The concentration of calcium used was 10μm, whereas the concentrations of ruthenium red and vanadate were 2.5μm and 4um, respectively. 5mm of sodium azide was removed from the medium for studying Ca^{2+} uptake in the absence of sodium azide.
* Significant from Control, $P<0.05$.

Table 6: Cardiac sarcoplasmic reticulum activities of marker enzymes and protein yield from control, diabetic and insulin treated diabetic rats.

	Control	Diabetic	Insulin treated
Protein yield, mg/g heart	1.75 ± 0.18	1.70 ± 0.25	1.80 ± 0.27
Glucose-6-phosphatase, uMol Pi/mg/hr	3.15 ± 0.17 (9.8)	2.99 ± 0.15 (10.1)	3.05 ± 0.10 (10.0)
Rotenone - insensitive NADPH cytochrome C reductase, nmol/mg/min	30.57 ± 2.04 (10.3)	32.05 ± 1.95 (10.5)	33.15 ± 2 (10.6)
Ouabain - sensitive Na^+ -K^+ ATPase, uMol Pi/mg/hr	1.58 ± 0.10 (0.70)	1.63 ± 0.12 (0.80)	1.60 ± 0.1 (0.75)
Cytochrome C oxidase, nmol/mg/min	45.17 ± 2.75 (0.6)	44.15 ± 2.30 (0.55)	43.39 ± 2 (0.50)

Values are means ± SE of 5 - 10 preparations per group. Values in parentheses represent the ratio of activities for marker enzymes in sarcoplasmic reticulum and respective homogenate.

Table 7: Cardiac sarcoplasmic reticulum ATPase activities in control, diabetic and insulin treated diabetic rats.

Group	Mg^{2+} -ATPase activity	Ca^{2+} -stimulated ATPase activity
1 Week		
Control	61.2 ± 6.71	10.31 ± 0.39
Diabetic	45.3 ± 4.90*	9.24 ± 0.30
10 Days		
Control	57.4 ± 7.25	9.91 ± 0.25
Diabetic	41.3 ± 3.14*	7.83 ± 0.32*
2 Weeks		
Control	62.7 ± 6.54	10.10 ± 0.33
Diabetic	47.1 ± 4.45*	6.95 ± 0.25*
4 Weeks		
Control	60.8 ± 7.15	9.88 ± 0.41
Diabetic	41.9 ± 3.85*	6.85 ± 0.33*
8 Weeks		
Control	56.8 ± 5.85	10.19 ± 0.44
Diabetic	39.2 ± 3.90*	6.04 ± 0.57*
Insulin Treated	63.8 ± 7.10	10.05 ± 0.55
12 Weeks		
Control	63.7 ± 5.25	9.85 ± 0.51
Diabetic	44.6 ± 4.29*	5.95 ± 0.39*

Values are means ± SE of 5 - 6 experiments. ATPase activities are expressed as umol Pi/mg/hr. * Significant from Control, $P<0.05$.

inhibitor of sarcolemmal Ca^{2+} pump at low concentrations, in the incubation medium did not affect Ca^{2+} uptake activities in SR preparations from control, diabetic and insulin treated diabetic rats. Furthermore, unlike the sarcolemmal preparations, 40 mM Na^{+} did not release Ca^{2+} in the Ca^{2+}-loaded SR preparations from control, diabetic or insulin treated diabetic rats. The extent of cross contamination of SR preparations by other subcellular organelles was determined by monitoring marker enzyme activities. From the data in Table 6, it can be seen that the SR protein yields from control, diabetic and insulin-treated preparations, were not different from each other. The activities of glucose-6-phosphatase and rotenone-insensitive NADPH cytochrome c reductase, well-known microsomal enzymes, showed that SR preparations were purified to an equal extent (~10 fold) in the control, diabetic and insulin treated diabetic groups with respect to heart homogenate activities (Table 6). On the other hand, ouabain-sensitive Na^{+}-K^{+}-ATPase, a sarcolemmal enzyme, and cytochrome c oxidase, a mitochondrial enzyme, in SR preparations were enriched about 0.8- and 0.6-fold with respect to the heart homogenate activities, respectively (Table 6). These results indicate that the SR preparations from control and experimental hearts were equally, but minimally contaminated with other subcellular organelles.

D. Sarcolemmal Enzymes

1. Marker enzymes and sarcolemma characterization

To clarify whether differential contamination was a confounding factor before any further investigations were conducted, activities of some selected marker enzymes were determined in the sarcolemmal membrane fractions from control, diabetic, and insulin treated diabetic rat

hearts (Table 8). The sarcolemmal yield was not different between the various groups and ranged between 1.1 and 1.21 mg protein/g tissue. The sarcolemmal preparations employed in this study were enriched by about 18 fold with respect to the heart homogenate Na^+-K^+-ATPase activities. Because ouabain-sensitive Na^+-K^+ ATPase activities in the sarcolemmal vesicles was on average 15-18% of the total Na^+-K^+-ATPase activity in all three preparations, the inside-outsided populations of the control, diabetic and insulin-treated sarcolemmal vesicles were similar (~82-85%). It should be noted that cytochrome c oxidase and rotenone-insensitive NADPH cytochrome c reductase activities of the sarcolemmal preparations shown in Table 8 suggest minimal contamination by mitochondria and SR in control, diabetic and insulin treated diabetic hearts.

2. Na^+-K^+ ATPase activity

Table 9 indicates that sarcolemmal Na^+-K^+-ATPase activity was significantly depressed in diabetic rats 10 days after alloxan administration. This depression persisted throughout the duration of the diabetic state; this depression was corrected in diabetic rats treated for 4 weeks with insulin. Na^+-K^+-ATPase activity of the sarcolemma from control, diabetic and insulin treated diabetic rats was studied by varying the concentration of Mg ATP in the incubation medium (Fig. 6). Increasing the concentration of Mg^{2+} ATP increased Na^+-K^+ ATPase activity in all groups, but Na^+-K^+-ATPase activity remained depressed compared with controls. Insulin administration normalized Na^+-K^+-ATPase activity in the presence of varying concentrations of Mg^{2+}-ATP.

3. Ca^{2+} pump activities

Sarcolemmal Ca^{2+}-stimulated ATPase, unlike Mg^{2+}-ATPase, was significantly depressed (17%) in diabetic sarcolemma preparations 10

Table 8: General characteristics of cardiac sarcolemmal membrane preparations from control, diabetic and insulin treated diabetic rats.

	Control	Diabetic	Insulin Treated
Sarcolemmal yield, mg protein/ g tissue	1.15 ± 0.20	1.21 ± 0.15	1.10 ± 0.14
Digitoxinigen-sensitive Na^+-K^+ ATPase, μmol/mg/min	24.6 (17.5)	16.3 (18.7)	24.0 (18.2)
Cytochrome C oxidase, nmol/mg/min	51 ± 5.1 (0.06)	49 ± 4.5 (0.51)	47 ± 5.0 (0.54)
Rotenone-insensitive cytochrome C reductase, nmol/mg/min	4.5 ± 0.4 (1.1)	4.1 ± 0.3 (1.1)	4.2 ± 0.5 (1.1)

Values are means ± SE of at least 8 preparations per treatment group. Values in parenthesis represent the ratio of activities for marker enzymes in the sarcolemma and respective homogenate.

Table 9: Influence of diabetes on Na^+ -K^+ ATPase and ouabain - sensitive ATPase activities in rat heart sarcolemmal membranes.

Group	Na^+ - K^+ ATPase activity	Ouabain - sensitive ATPase - activity
1 Week		
Control	23.7 ± 1.1	4.20 ± 0.85
Diabetic	20.1 ± 0.9	3.55 ± 0.90
10 Days		
Control	22.1 ± 0.7	2.51 ± 0.50
Diabetic	18.0 ± 0.8*	3.25 ± 0.77
2 Weeks		
Control	23.2 ± 1.2	2.95 ± 0.54
Diabetic	16.8 ± 1.3*	1.99 ± 0.79
4 Weeks		
Control	25.6 ± 0.9	4.41 ± 0.83
Diabetic	17.1 ± 0.7*	3.44 ± 0.81
8 Weeks		
Control	25.7 ± 1.0	4.01 ± 0.69
Diabetic	16.5 ± 1.2*	3.20 ± 0.74
Insulin treated	25.0 ± 1.3	4.28 ± 0.65
12 Weeks		
Control	24.8 ± 1.5	3.95 ± 0.84
Diabetic	17.3 ± 1.1*	3.56 ± 0.95

Values are means ± SE of 5 - 7 experiments. The ATPase activities are expressed as umol Pi/mg/hr. Sarcolemma treated with 0.2 mg deoxycholate/mg sarcolemmal protein to expose all sites for enzymatic reaction; the activity was completely inhibited by 2mM ouabain. Ouabain sensitive without any pretreatment refers to enzyme activity inhibited by 2mM ouabain. * Significant from Control, $P<0.05$.

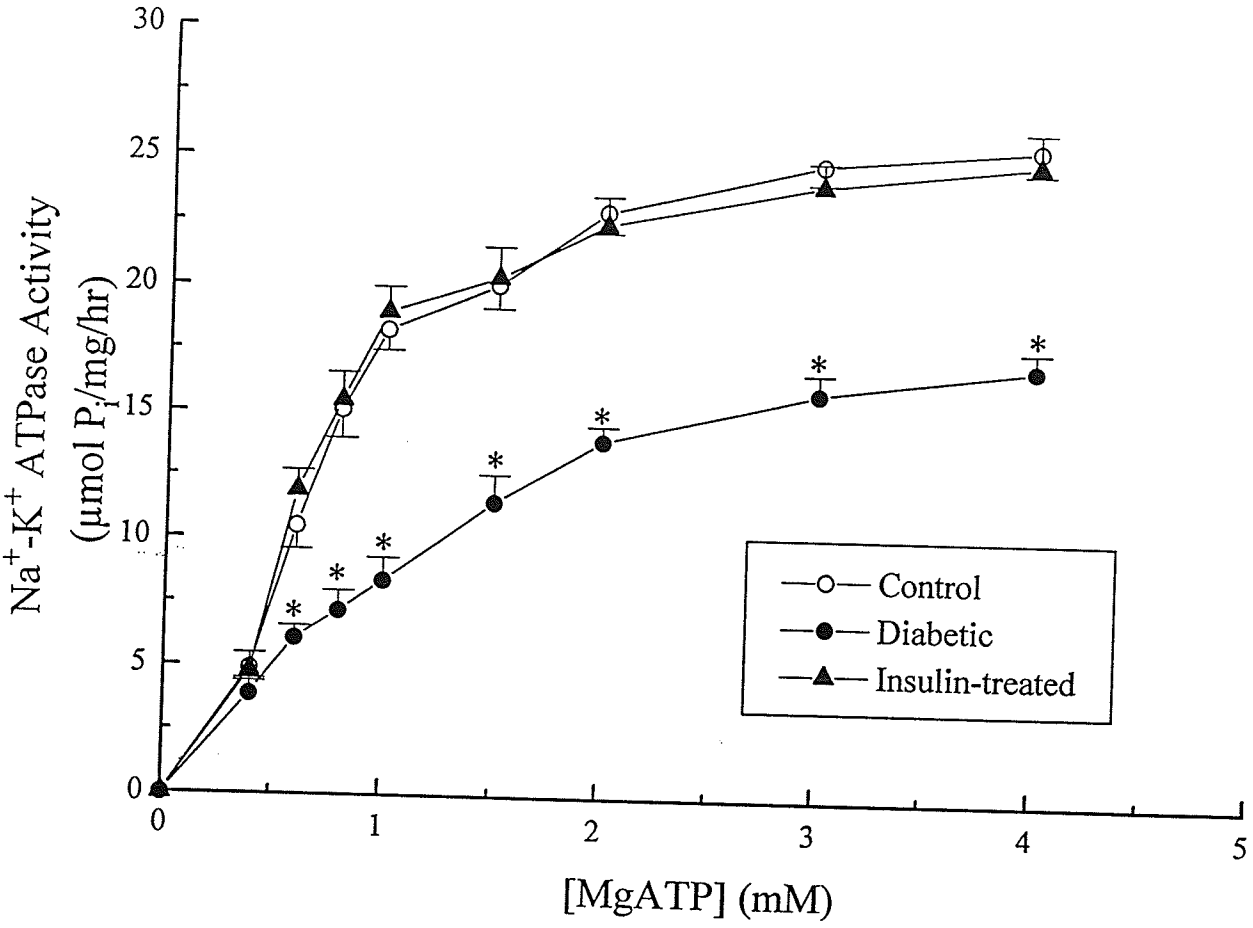

Figure 6. Cardiac sarcolemmal Na^+-K^+ ATPase activity at different concentrations of MgATP in control, diabetic, and insulin-treated diabetic rats. Values are means ± SE of 4 to 6 experiments. *Significantly different ($P < 0.05$) from control.

days after alloxan administration (Table 10). Insulin administration completely reversed the depression in Ca^{2+}-stimulated ATPase activity associated with the diabetic state. Since sodium azide was found to exert no significant effect on sarcolemmal Mg^{2+}-ATPase and Ca^{2+}-stimulated ATPase activities (Table 11), the observed changes in the Ca^{2+}-stimulated ATPase activity does not appear to be due to mitochondrial contamination. It should be pointed out that unlike sarcoplasmic reticulum, the Ca^{2+}-stimulated activities of all the sarcolemmal preparations were all depressed similarly by increasing the vanadate concentrations from 0.5 to 4 uM.

ATP-dependent sarcolemmal Ca^{2+} uptake was also depressed by about 25% in diabetic rats 14 days after the administration of alloxan (Table 12). In addition, in comparison to control preparations, diabetic sarcolemmal ATP-dependent Ca^{2+} uptake was depressed even when the concentration of free calcium (pCa) was increased in the incubation medium (Fig. 7). Insulin administration reversed the depressions seen in Fig. 7 and Table 12.

4. Na^{+}-Ca^{2+} exchange activity

Another set of experiments was concerned with the measurement of Na^{+}-dependent Ca^{2+}-uptake in sarcolemmal vesicles isolated from hearts after different times of inducing diabetes. Table 13 shows that Na^{+}-Ca^{2+} exchange activity in diabetics was depressed by ~42% compared to controls 2 weeks after alloxan administration. Figure 8 indicates that both rate and capacity of Na^{+}-dependent Ca^{2+} uptake were depressed from in diabetic sarcolemmal preparations. Na^{+}-dependent Ca^{2+} uptake was also determined by employing different concentrations of Ca^{2+}, and the results shown in Figure 9 reveal a

Table 10: Influence of diabetes on Ca^{2+} -stimulated, Mg2+ - dependent ATPase activity in rat heart sarcolemmal membranes.

Group	Mg^{2+} -dependent ATPase activity	Ca^{2+} -stimulated ATPase activity
1 Week		
Control	120.5 ± 10.6	19.15 ± 1.85
Diabetic	115.7 ± 8.7	17.18 ± 1.19
10 Days		
Control	99.7 ± 12.2	18.65 ± 1.54
Diabetic	103.5 ± 11.6	15.49 ± 1.15*
2 Weeks		
Control	117.1 ± 13.2	19.25 ± 1.07
Diabetic	105.3 ± 10.4	13.10 ± 0.99*
4 Weeks		
Control	95.1 ± 12.6	18.91 ± 1.43
Diabetic	108.4 ± 11.4	12.95 ± 1.21*
8 Weeks		
Control	112.0 ± 9.9	19.35 ± 1.01
Diabetic	102.6 ± 13.1	13.41 ± 1.22*
Insulin treated	120.5 ± 14.2	20.2 ± 1.57
12 Weeks		
Control	101.5 ± 12.5	18.51 ± 1.27
Diabetic	98.4 ± 8.5	12.67 ± 0.98*

Values are means ± SE of 5 - 7 experiments and are expressed as umol Pi/mg/hr. * Significant from Control, $P<0.05$.

Table 11: Rat heart sarcolemmal membrane Ca^{2+} -stimulated and Mg^{2+} -dependent ATPase activities in the absence or presence of sodium azide.

Group	Mg^{2+}-dependent ATPase activity	Ca^{2+}-stimulated ATPase activity
In absence of sodium azide		
Control	107.1 ± 9.4	17.95 ± 1.27
Diabetic	115.2 ± 10.5	13.02 ± 0.95*
Insulin treated	128.7 ± 13.4	18.26 ± 1.41
In presence of 5mM sodium azide		
Control	95.1 ± 12.6	18.91 ± 1.43
Diabetic	108.4 ± 11.4	12.95 ± 1.21*
Insulin treated	120.5 ± 14.2	20.2 ± 1.57

Values are means ± SE of 4 - 7 experiments and are expressed as umol Pi/mg/hr. * Significant from Control, $P<0.05$.

Table 12: Influence of diabetes on ATP -dependent Ca^{2+} uptake in rat heart sarcolemmal membranes.

Group	ATP -dependent Ca^{2+} uptake	Non - specific Ca^{2+} uptake
1 Week		
Control	19.1 ± 0.94	3.05 ± 0.74
Diabetic	17.4 ± 0.90	2.85 ± 0.83
10 Days		
Control	18.9 ± 1.0	2.85 ± 0.59
Diabetic	16.0 ± 0.80	2.94 ± 0.71
2 Weeks		
Control	18.5 ± 0.85	3.25 ± 0.87
Diabetic	14.0 ± 0.90*	2.25 ± 0.75
4 Weeks		
Control	20.1 ± 1.1	2.58 ± 0.71
Diabetic	13.2 ± 1.8*	1.99 ± 0.80
8 Weeks		
Control	18.1 ± 1.5	2.11 ± 0.91
Diabetic	12.1 ± 1.7*	1.55 ± 0.55
Insulin Treatment	18.8 ± 1.0	2.47 ± 0.77
12 Weeks		
Control	18.7 ± 1.2	2.78 ± 0.99
Diabetic	13.2 ± 0.9*	1.95 ± 0.66

Values are means ± SE of 5 - 7 experiments and are expressed as nmol Ca^{2+}/mg/min. ATP -dependent Ca^{2+} uptake was measured in the presence of 10uM free calcium. * Significant from Control, $P<0.05$.

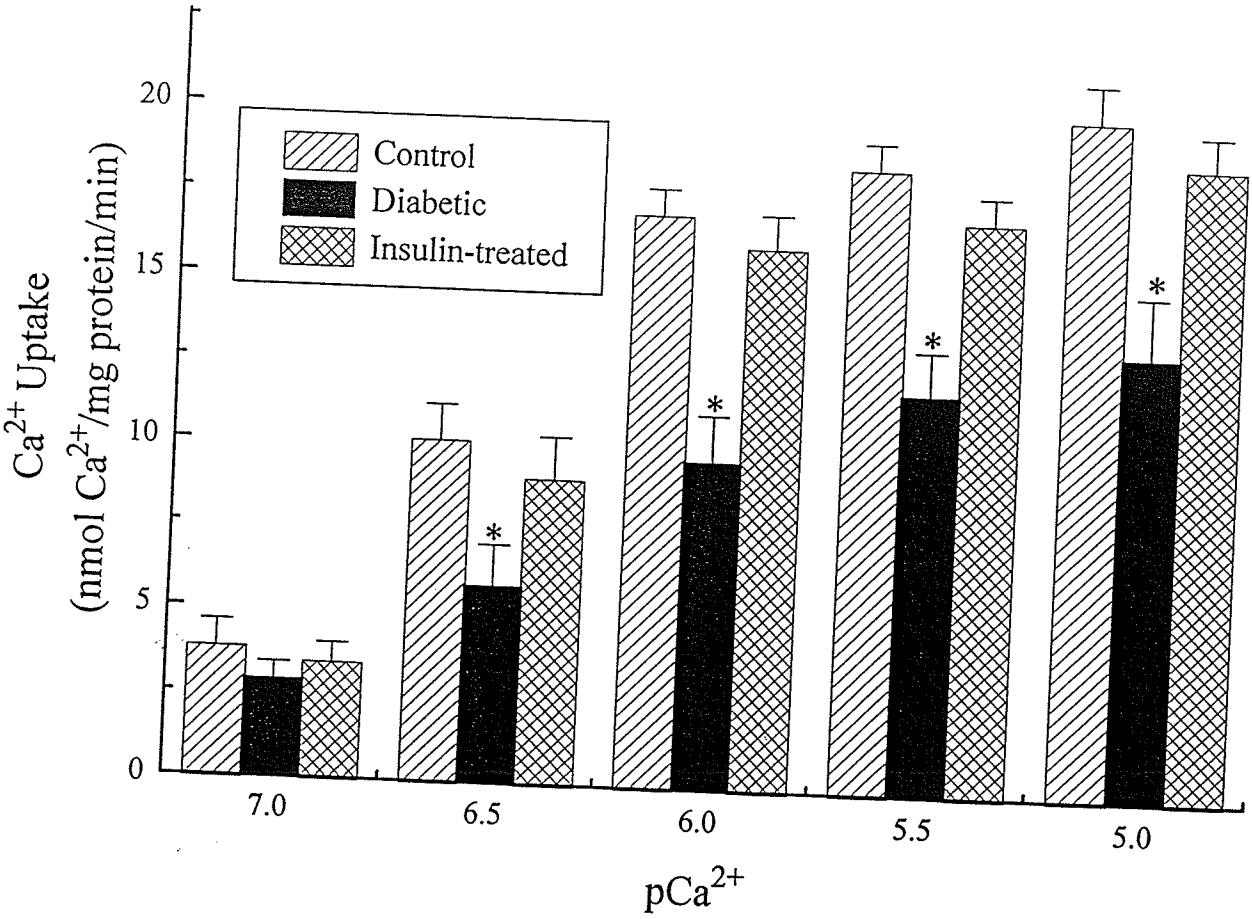

Figure 7. Cardiac sarcolemmal ATP-dependent Ca^{2+} uptake at different concentrations of Ca^{2+} in control, diabetic and insulin-treated diabetic rats. Values are means ± SE of 5 to 7 experiments. *Significantly different ($P < 0.05$) from control.

Table 13: Influence of diabetes on Na^+-Ca^{2+} exchange in rat heart sarcolemmal membranes.

Group	Na^+-dependent Ca^{2+} uptake	Non-specific uptake
1 Week		
Control	5.04 ± 0.95	1.59 ± 0.61
Diabetic	4.15 ± 0.81	1.35 ± 0.55
10 Days		
Control	4.95 ± 0.89	1.71 ± 0.75
Diabetic	3.65 ± 0.97	1.58 ± 0.49
2 Weeks		
Control	4.85 ± 0.71	1.50 ± 0.62
Diabetic	2.80 ± 0.55*	1.29 ± 0.57
4 Weeks		
Control	5.20 ± 0.85	1.85 ± 0.77
Diabetic	2.99 ± 0.67*	1.58 ± 0.81
8 Weeks		
Control	4.97 ± 0.80	1.95 ± 0.41
Diabetic	2.75 ± 0.63*	1.77 ± 0.45
Insulin Treatment	5.14 ± 0.85	1.80 ± 0.64
12 Weeks		
Control	4.91 ± 0.76	1.78 ± 0.71
Diabetic	2.69 ± 0.58*	1.90 ± 0.80

Values are means ± SE of 5 - 7 experiments and are expressed as nmol Ca^{2+}/mg/2sec. Final Ca^{2+} concentration in all experiments was 40uM. * Significant from Control, $P<0.05$.

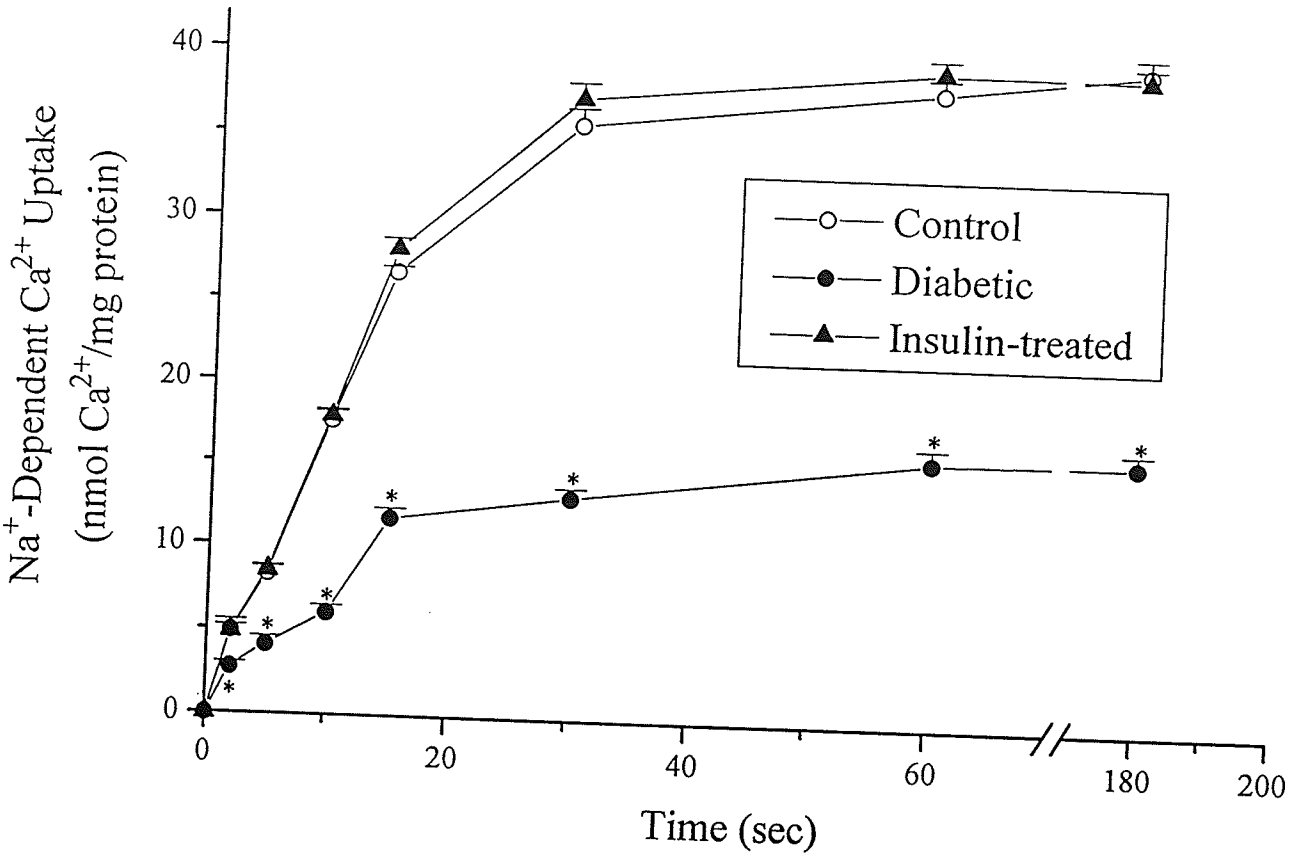

Figure 8. Cardiac sarcolemmal Na^+-dependent Ca^{2+} uptake activity over various times of incubation in control, diabetic and insulin-treated diabetic rats. Na^+-dependent Ca^{2+}-uptake activity was measured in the presence of 40 uM Ca^{2+}. Values are means ± SE of 5 to 6 experiments. *Signficantly different ($P < 0.05$) from control.

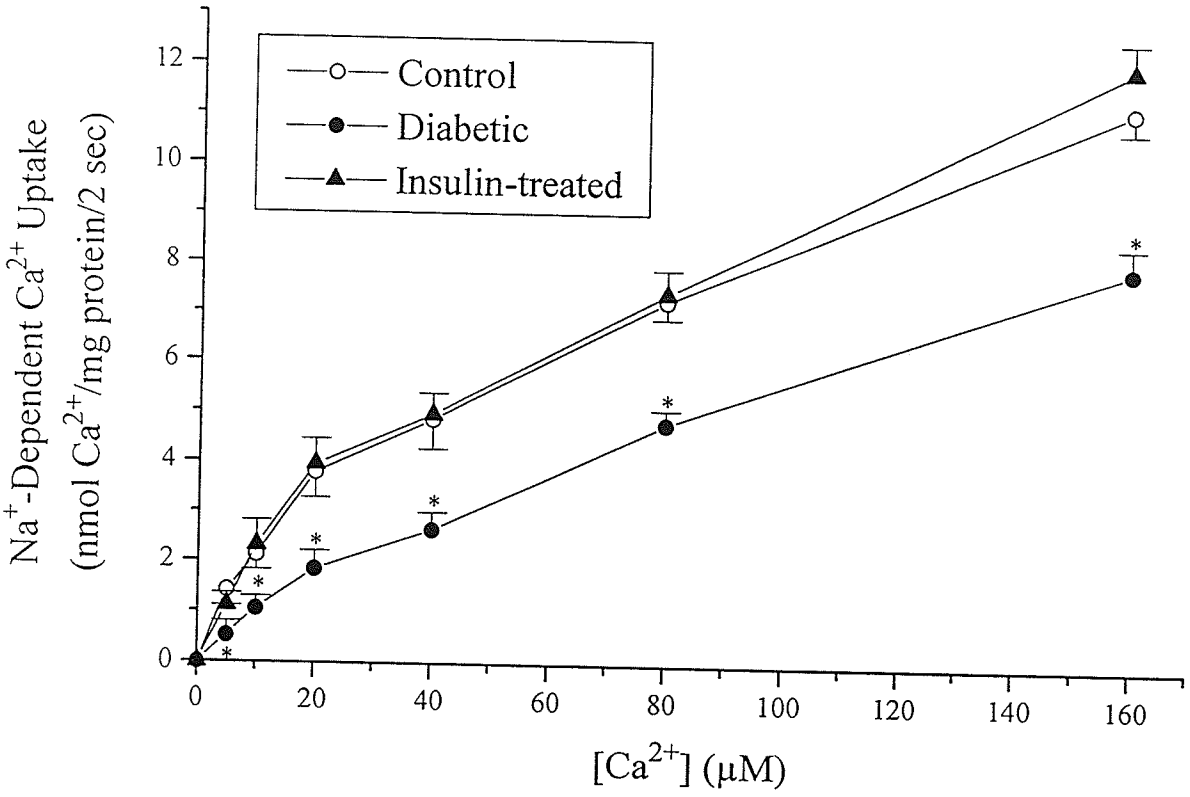

Figure 9. Cardiac sarcolemmal Na^+-dependent Ca^{2+}-uptake activity at different concentrations of Ca^{2+} in control, diabetic and insulin-treated diabetic rats. Values are means ± SE of 5 to 6 experiments. *Significantly different ($P < 0.05$) from control.

depression in the activity at virtually all concentrations of Ca^{2+}. There were no differences in the non-specific Ca^{2+} binding amongst all sarcolemmal preparations in these above experiments. Insulin administration reversed the observed depressions in both the time course and Ca^{2+}-dependency studies (Figs. 8 & 9).

E. Molecular Biology Studies

1. Alpha-myosin

The data concerning alpha-myosin heavy chain gene expression in left ventricles obtained from rats 2, 3, 5 and 6 weeks after alloxan administration in comparison to their age-matched controls are presented in Figure 10, with a typical Northern blot depicted in Figure 11. The relative intensity of the mRNA signal (normalized to GAPDH) was dramatically depressed at 2 weeks after alloxan administration. This depression was further maintained 3 weeks after alloxan administration. By 5 and 6 weeks after alloxan administration, however, the relative intensity of the transcript for the mRNA signal in diabetics was not significantly different with respect to the age-matched control groups at these time points.

2. SERCA2

The results for SERCA2 expression in left ventricles obtained from rats 2, 3, 5 and 6 weeks after alloxan administration in comparison to their age-matched controls are presented in Fig. 12, with a typical Northern blot depicted in Figure 11. SERCA2 expression 2 weeks after alloxan administration in diabetics was not significantly altered in comparison to the control group. However, by 3 and 5 weeks after alloxan administration, SERCA2 expression was significantly elevated with respect to the control group. This small elevation in expression at both these

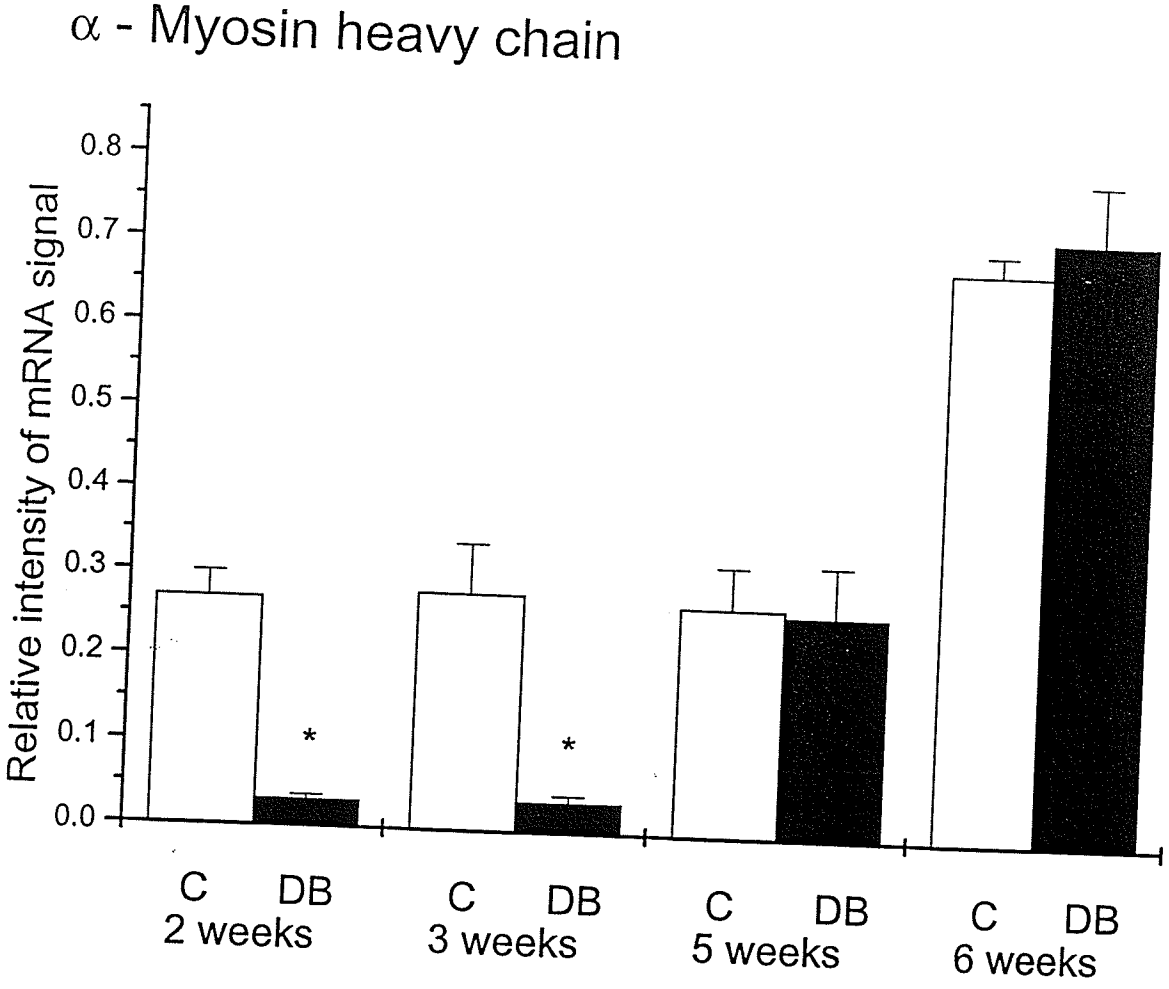

Figure 10: mRNA abundance of cardiac α-myosin heavy chain gene in hearts of alloxan-diabetic and age-matched control rats. The relative intensity of mRNA signal was expressed as the ratio of densitometric intensity of α-myosin heavy chain signal/Glyceraldehyde phosphate dehydrogenase (GAPDH) signal. Values are represented as means $\pm$ SE of 4-6 experiments. * Significant from control, $P \leq 0.05$.

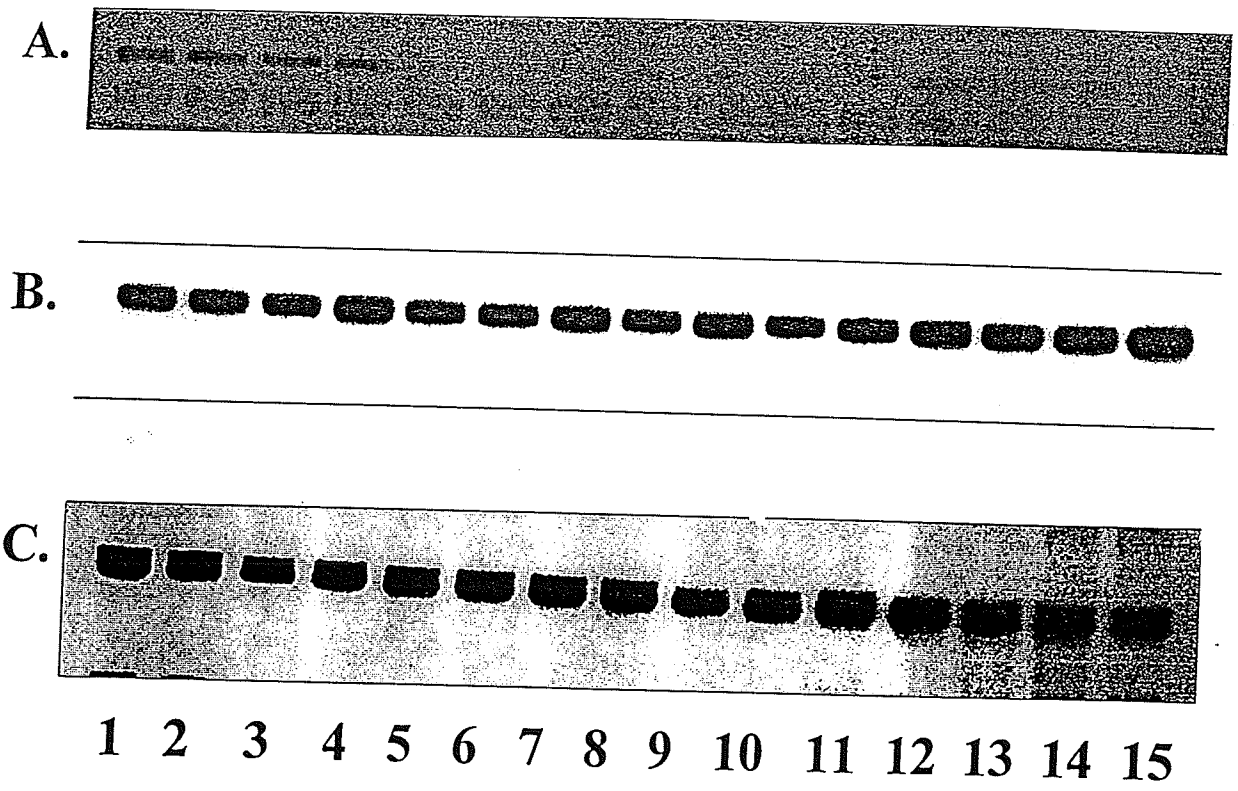

Figure 11. Representative Northern blots using 40 ug total RNA extracted from either control or alloxan diabetic rat hearts. A. cardiac α-myosin heavy chain gene. B. cardiac sarcoplasmic reticular Ca^{2+} ATPase gene (SERCA2). C. cardiac GAPDH gene. 1-4: control samples, 5 - 9: 2 - week diabetic samples, 10 - 15: 3 - week diabetic samples.

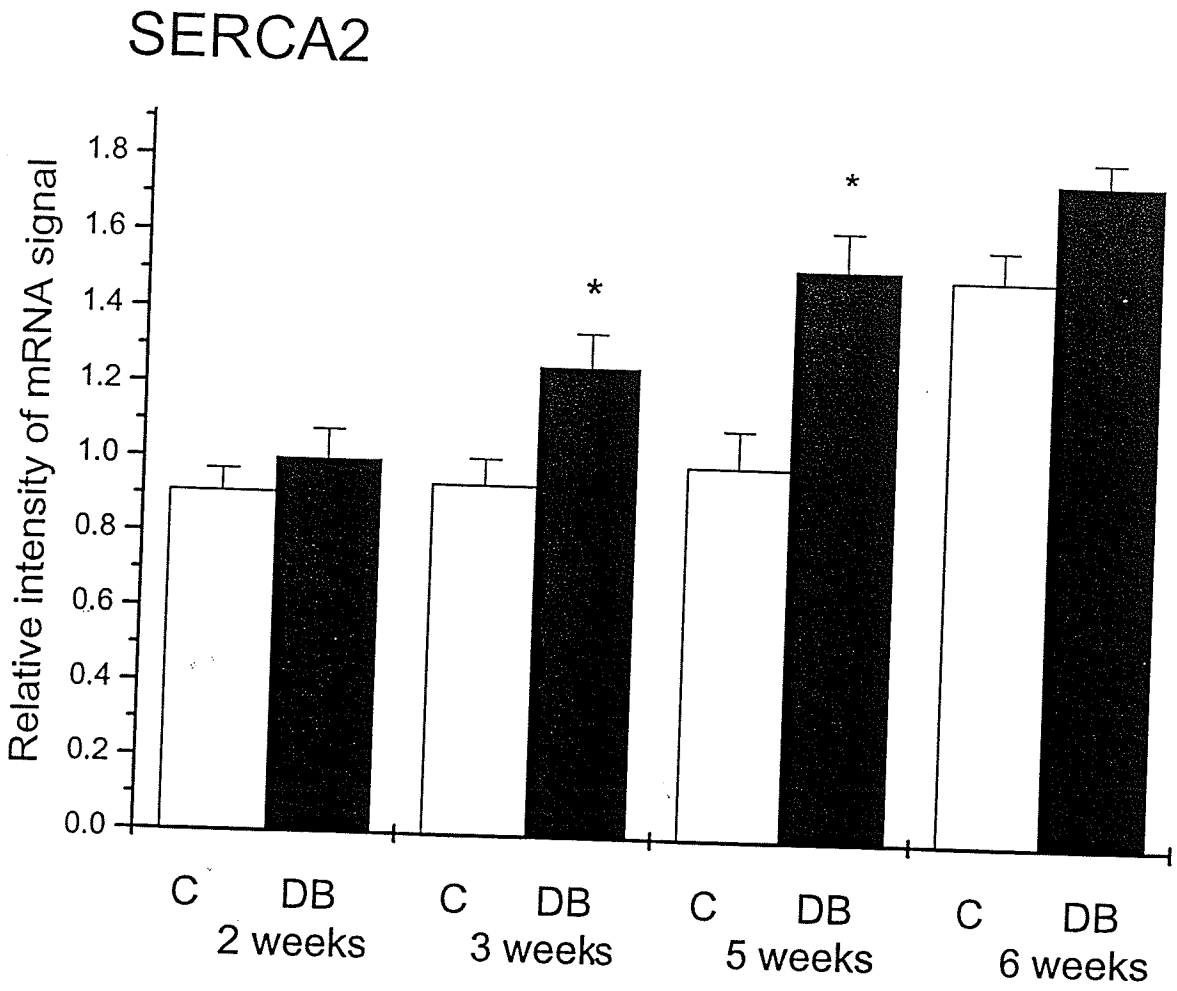

Figure 12: mRNA abundance of cardiac sarcoplasmic reticular Ca^{2+} ATPase gene (SERCA2) in hearts of alloxan - diabetic and age-matched control rats. The relative intensity of mRNA signal was expressed as the ratio of densitometric intensity of SERCA2 signal/GAPDH signal. Values are represented as means ± SE of 4-6 experiments. * Significant from control, $P \leq 0.05$.

time points accounted for about a 30% increase over the control value. Six weeks after alloxan administration, there was no significant change in expression of the SERCA2 in the diabetic ventricles in comparison to the control ventricles.

3. Alpha$_1$-Subunit - Na^+-K^+ ATPase

Figure 13 illustrates that 2 to 5 weeks after alloxan administration there was no significant change in the expression of the alpha$_1$-Na^+-K^+ ATPase transcript in the diabetic ventricles with respect to the control ventricles. Figure 14 represents a typical Northern blot. However, in diabetic ventricles 6 weeks after alloxan administration, a significant depression was observed in the expression of the alpha$_1$-Na^+-K^+ ATPase subunit in the diabetic ventricles. We also examined the expression of the Na^+-Ca^{2+} exchange transcript two and three weeks after alloxan administration (data not shown). In these studies (n=2), we could detect no statistically significant change in this transcript expression between the control and diabetic ventricles. No effort was made to examine the expression of sarcolemmal Ca^{2+} pump ATPase as the appropriate probe for this purpose was not available to us.

F. General Characteristics and Subcellular Changes in Experimental Rabbits

In order to examine if the changes observed in cardiac subcellular organelles are limited to the rat model of diabetes, the alloxan-induced diabetes in rabbit was employed to study changes in the heart. Table 14 shows the general characteristics of the control and diabetic rabbits. Body weights and heart weights were significantly depressed in diabetic rabbits compared to control rabbits, 12 weeks after alloxan administration. A marked increase in plasma glucose and cholesterol

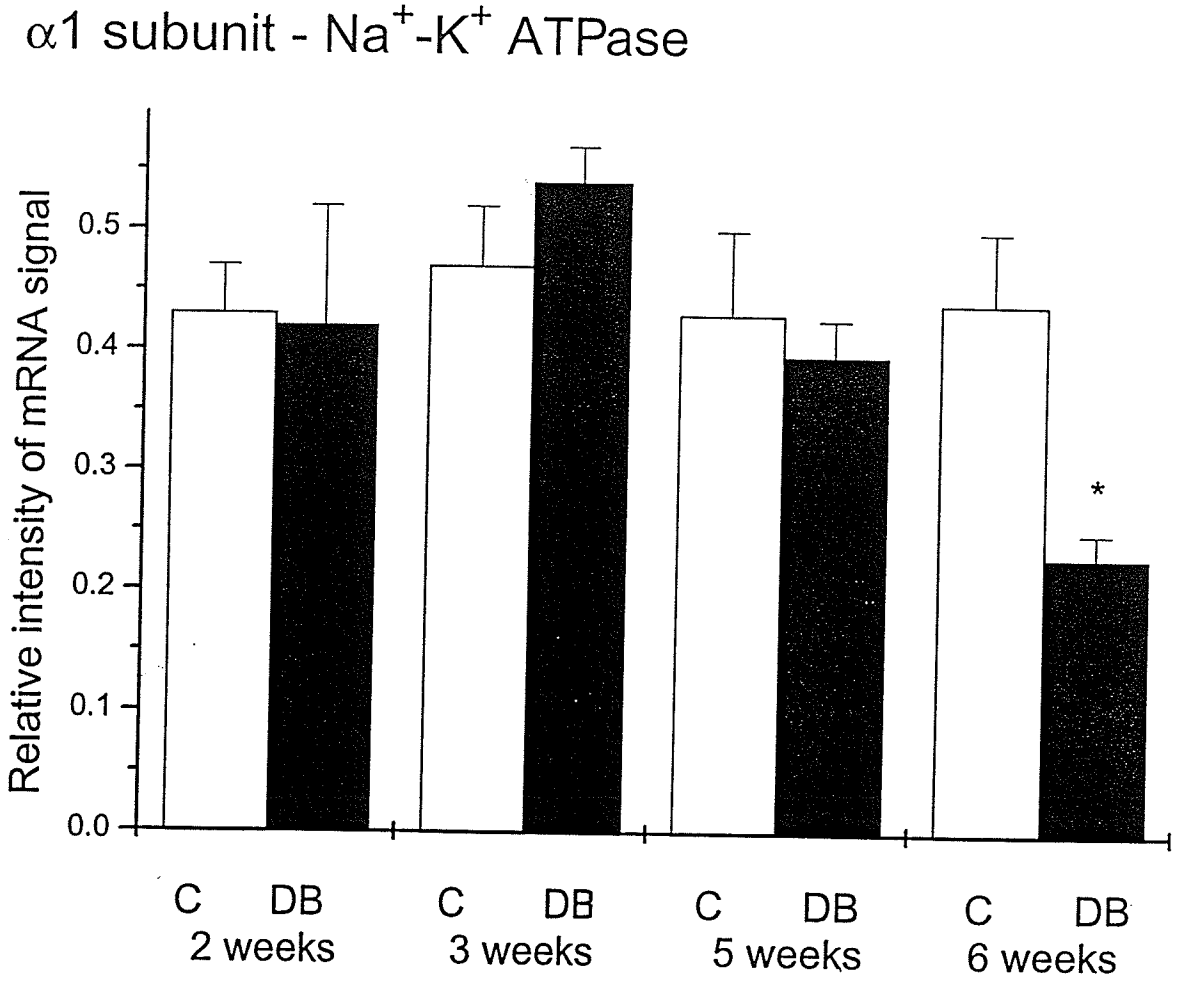

Figure 13: mRNA abundance of α1-subunit of cardiac sarcolemmal Na^+-K^+ ATPase gene in hearts of alloxan - diabetic and age-matched control rats. The relative intensity of mRNA signal was expressed as the ratio of densitometric intensity of α1-Na^+-K^+ ATPase signal/GAPDH signal. Values are represented as means $\pm$ SE of 4-6 experiments. * Significant from control, $P \leq 0.05$.

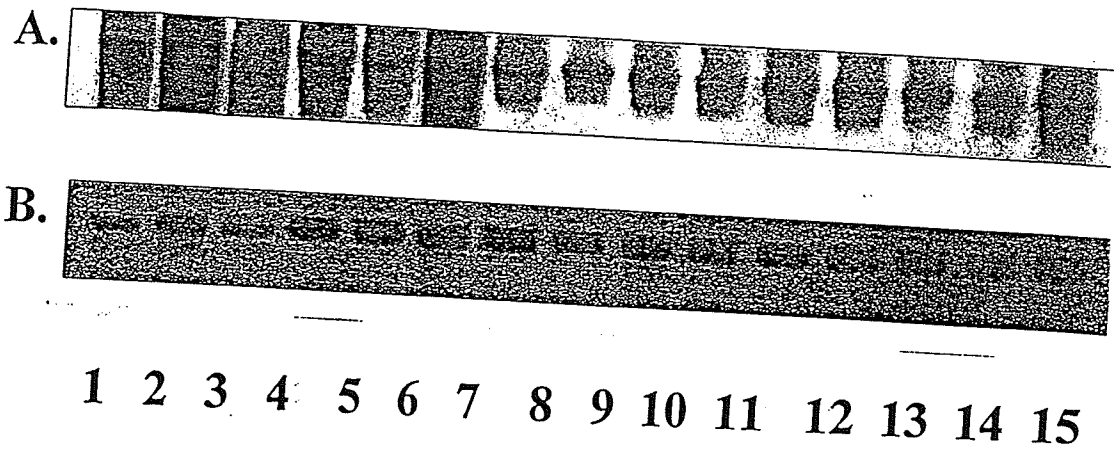

Figure 14. Representative Northern blots using 40 ug total RNA extracted from either control or alloxan diabetic rat hearts. A. α1 - subunit of cardiac sarcolemmal Na^+-K^+ ATPase gene. B. cardiac GAPDH gene. 1 - 3: 5 week control samples, 4 - 6: 5 - week diabetic samples, 7 - 9: 6 - week control samples, 10 - 15: 6 - week diabetic samples.

Table 14: General characteristics of control and diabetic rabbits.

	Control	Diabetic
Body Weight, kg	4.50 ± 0.25	3.10 ± 0.15*
Heart Weight, g	6.53 ± 0.51	3.60 ± 0.62*
Plasma Concentrations:		
Glucose (mg/dL)	133.5 ± 11.3	457.9 ± 40.6 *
Insulin (mu/l)	20.3 ± 2.1	15.1 ± 1.0 *
Cholesterol (mmol/dL)	2.06 ± 0.25	4.81 ± 0.35*

Values are means ± SE of 5 - 6 animals per experimental group.
* Significant from Control, $P<0.05$.

levels was seen in experimental rabbits whereas plasma insulin levels were decreased. Table 15 shows that myofibrillar Ca^{2+}-stimulated ATPase was significantly depressed (32%) in diabetic rabbits compared to controls; however, myofibrillar Mg^{2+}-stimulated ATPase was unaffected. In diabetic rabbits, both Ca^{2+}-stimulated and Mg^{2+}-stimulated SR ATPase activity were reduced in comparison with control rabbits. Table 16 reveals that only the Na^{+}-dependent Ca^{2+} exchange activity was severely depressed (41%) in the diabetic rabbits compared to the control; neither the Na^{+}-K^{+}-ATPase activity nor the Ca^{2+} pump indices were affected.

Table 15: Influence of diabetes on myofibrillar and sarcoplasmic reticulum ATPase activity in rabbit hearts.

	Myofibrillar ATPase Activity (umol Pi/mg/hr)		Sarcoplasmic Reticulum ATPase Activity (umol Pi/mg/5 min)	
Group	Mg^{2+}-stimulated	Ca^{2+}-stimulated	Mg^{2+}-stimulated	Ca^{2+}-stimulated
Control	1.80 ± 0.13	8.18 ± 0.23	6.6 ± 0.2	0.81 ± 0.07
Diabetic	1.59 ± 0.18	5.60 ± 0.46*	4.2 ± 0.3*	0.54 ± 0.05*

Values are means ± SE of 4 - 7 experiments. * Significant from Control, $P<0.05$.

Table 16: Influence of diabetes on rabbit heart sarcolemmal enzymes.

	Control	Diabetic
Na+ -dependent Ca^{2+} uptake (nmul Ca^{2+}/mg/2sec)	3.50 ± 0.56	2.05 ± 0.50*
Na^{+}-K^{+}ATPase (umol Pi/mg/hr)	9.8 ± 1.0	7.8 ± 0.93
ATP -dependent Ca^{2+} uptake (nmol Ca^{2+}/mg/5min)	12.1 ± 2.0	9.9 ± 1.85
Ca^{2+} -stimulated ATPase (umCa^{2+}) (umol Pi/mg/hr)	6.95 ± 1.0	5.45 ± 0.8
Mg^{2+} -stimulated ATPase (umol Pi/mg/hr)	125 ± 10.5	131 ± 15.2

Values are means ± SE of 4 - 6 experiments. * Significant from Control, $P<0.05$.

V. DISCUSSION

A. Alloxan-Diabetic Rat Model: General Characteristics and Hemodynamic Alterations

One of the leading causes of mortality in diabetics is myocardial disease since cardiovascular disease accounts for nearly 80% of all deaths in diabetic patients (11) and the frequency of diabetic deaths is two to three times that of the non-diabetic population (63). Though originally thought to occur as a result of atherosclerosis, various studies have shown that heart disease can occur in the absence of atherosclerosis, suggesting a diabetic cardiomyopathy, without ischemic involvement or ischemic processes. Studies on diabetic cardiomyopathy have employed a wide variety of experimental models. Several rat models such as streptozotocin-induced diabetes and alloxan-induced diabetes are presently available for the study of the pathophysiology of organ dysfunction, including cardiomyopathy. In the past, the streptozotocin-induced diabetic rat model has been predominantly used by various investigators for investigating functional, structural and biochemical changes during the development of cardiomyopathy. The results reported with respect to cardiac alterations in streptozotocin in rats have been similar to those obtained with alloxan-induced diabetes in rats.

The alloxan-induced rat model of diabetes used in this study has been well documented since it was first described by Dunn and co-workers in 1943 (111), and later characterized by Lazarow and Palay (524) as well as Lazarow (525). This model produces a constellation of both direct and indirect changes which closely mimics the disease in humans. The alloxan model, as with the STZ model, demonstrates cardiac dysfunction, ultrastructural and subcellular and biochemical defects in the absence of

vascular changes in a stable model of chronic, poorly controlled Type I diabetes. In this study, we monitored the diabetic state by examining changes in plasma and urine glucose, plasma insulin, in addition to measuring body weight, ventricular weight, ventricular weight-to-body weight ratios and urine ketones. In accordance with numerous studies using either alloxan- or STZ-induced diabetic animals, the plasma glucose concentrations ($\geq$495 mg/dl) were greatly elevated and plasma insulin levels were depressed in comparison with age-matched control rats. The dramatic decrease in body weights, ventricular weights and elevated ventricular-to-body weight ratios are reflective of the metabolic derangements associated with experimental diabetes and are consistent with the very many rat studies using this or the STZ-model of diabetes. These altered characteristics were subsequently normalized by chronic insulin administration (3 U/day) for 4 weeks.

Previous work has established that cardiac dysfunction accompanies diabetes mellitus (251,259), and cellular or subcellular causes for dysfunction have been identified. However, there are two problems when such an information is applied to overall cardiovascular function in the diabetic condition. First, most of the studies in the past have dealt primarily with cardiac functional evaluation in the isolated heart. These type of studies provide evidence that intrinsic cardiac lesions are effected by diabetes but do not allow evaluation of potential compensatory adaptation or additive defects which may occur when the heart is functioning as part of an intact, integrated cardiovascular system. Second, many of the cardiovascular dysfunctions associated with diabetes mellitus are known to be time-dependent. Some lesions develop rapidly while others are not functionally detectable for extended periods

of time. The time-dependent nature of developing cardiovascular lesions would also be expected to influence the integrative nature of hemodynamic status in those with diabetes. The integrative nature of cardiovascular function, including assessment of hemodynamic status (in anesthetized diabetic, age-matched controls, and insulin treated rats) and time-dependent aspects of the biochemical responses of subcellular organelles and molecular changes in diabetic rats comprise the experimental nature of this present study.

Bradycardia is commonly observed in experimental models of diabetes (322,527-530). Our results show a gradual (non-statistically significant) decrease in heart rate at 10 days (365 beats/min) to a statistically significant reduced heart rate (313 beats/min) at 4 weeks in diabetic rats; this represents a 14% depression with respect to control. A decreased heart rate in the STZ model, as with the alloxan model, appears to be progressive in nature, reaching 277 beats/min (or 70% of control). The underlying mechanisms of this bradycardia are obscure, and it appears limited to drug-induced diabetes in rats (322,527-530) since in human diabetics, heart rates remain unchanged (274,278). Saverese and Berkowitz (422) were the first to report a decrease (28%) in the number of cardiac ß-adrenoceptors and it was suggested that this reduction in receptor number might have contributed to the bradycardia seen in their animals. Since these early findings, there have been several reports of diminished cardiac ß-adrenergic receptor number, with no change in affinity, in cardiac tissue taken from 4 days to 10 weeks after treatment with alloxan or STZ. From these numerous studies, it is notable that there doesn't appear to be any consistency with regard to the time of onset of changes in ß-

adrenoceptor numbers, nor does there exist a clear relationship between these changes and the time of onset of bradycardia, which may occur within 4 days of STZ-treatment (431). In addition, Jackson and Carrier (527) have reported an increase in parasympathetic outflow in the diabetic rat. In the present study, however, ß-adrenergic receptor number at early or late time points were not measured nor was parasympathetic outflow characterized. Furthermore, a conclusive mechanism for the regulation of the ß-adrenergic receptors in diabetes is far from clear, and further studies are warranted for this type of investigation in alloxan-induced diabetes.

This study demonstrates that at 4 weeks, diabetic animals also had depressed left ventricular systolic pressure and depressed mean arterial pressure. Our results with respect to depressed heart rates and depressed left ventricular systolic pressure four weeks after alloxan administration are in very good agreement with the in vivo hemodynamic studies conducted by Rosen et al (531) using STZ-induced diabetic rats. These investigators have reported a decrease in in vivo heart rate and left ventricular systolic pressure by about 13% and 16%, whereas in our study depressions of 19% and 15% were observed, respectively.

In our present study, we report a significant fall in the rate of relaxation (-dP/dt) as early as two weeks after alloxan administration, followed by a subsequent fall in rate of contraction (+dP/dt) and enhanced left ventricular end diastolic pressure compared with controls. These alterations are similar to those reported in numerous isolated cardiac preparations from diabetic animals and also in in vivo studies. Such hemodynamic abnormalities were associated with chronic diabetes and were normalized by controlling the hyperglycemia and hypoinsulinemia by

subcutaneous insulin administration for four weeks.

In general, the hemodynamic findings with our diabetic hearts in vivo demonstrate a significant depression in contractile performance of the heart and these correspond to those obtained in human diabetics (278). All indices of force generation in the hearts of diabetic animals have been reported in a large number of studies to be subnormal. The ability to generate a peak amount of force or pressure is depressed. In addition, the rate of force or pressure development was slower (119-121,123,134-136,318-327). Affected indices of force generation reported in these studies from diabetic hearts include augmentation of time to peak tension, time to peak shortening, and time to peak shortening velocity. Alterations in end-diastolic volume have also been reported (120,251). The change in the ability of the heart to relax during diabetes can even be more dramatic than the changes in force generation as reported in this study. We report an earlier depression in -dP/dt compared with a later fall in +dP/dt. The depression in -dP/dt represents a 16% decrease; it then progresses to a 58% decline by 12 weeks after alloxan administration. The depression in +dP/dt at 4 weeks represents a 38% decline and continues to decline 54% compared with controls.

Our report of an early fall in -dP/dt in the alloxan model illustrates that the presence of this diabetic state slows the rate of relaxation and prolongs the amount of time it takes to dissipate tension. Changes in tension generation and relaxation in papillary muscles from diabetic and control animals were first demonstrated from an early study by Fein et al (134). When one considers various indices of relaxation affected in hearts from diabetic animals, several studies have

overwhelmingly shown: attenuation of peak velocity of relaxation, -dP/dt as well as -dT/dt, and augmentation of such parameters as time to peak relaxation velocity, time for peak tension to fall by 50%, and time to peak rate of tension fall (119,120,123,134,320,321).

Our hemodynamic studies are in general agreement with those reported earlier indicating that the diabetic hearts are in a compromised position, illustrating changes in left ventricular end diastolic pressure, rates of contraction and relaxation, and depressed heart rates. The finding that -dP/dt declines before +dP/dt in vivo in the alloxan model is in contrast with in vivo studies using STZ-induced diabetic rats which have shown concurrent changes in both of these parameters. The model of diabetes used in this study, severity of diabetes, or progressive ultrastructural changes, and microvascular dysfunction could be possible factors predisposing diabetic rats to an early decline in the rate of relaxation. It is also not clear at this time whether the ketotic nature of alloxan diabetes vs. the STZ model may influence the early changes noted in our study vs. those utilizing a non-ketotic and perhaps milder diabetic model.

B. Subcellular and Biochemical Changes

Since cardiac function is ultimately determined by the control of cellular ion movements by different membrane systems such as the SR, sarcolemma as well as interaction of contractile and regulatory proteins, studies were thus undertaken to examine biochemical explanations for the observed depression in heart function in diabetes. Our study, in agreement with many others (135,215,355-358) show that myofibrillar Ca^{2+}-stimulated ATPase and basal (Mg^{2+}-stimulated) ATPase levels were depressed ~30% 4 weeks after alloxan administration. This also

coincided with the observed depression in +dP/dt. In addition, the depression in Ca^{2+}-stimulated ATPase activity of cardiac myofibrils from diabetic animals was observed over a full range of physiologically relevant Ca^{2+} concentrations (pCa=5-7). Our biochemical results were not confounded by contamination of myofibrils with other subcellular membranes, since myofibrillar ATPase activities were insensitive to ouabain and sodium azide, myofibrillar preparations did not display oxalate-supported Ca^{2+}-uptake activity. These observations indicate that sarcolemma, mitochondria, and sarcoplasmic reticulum were absent from our myofibrillar preparations and that the relative purity of our preparations was apparently similar between all groups in this study.

Reduced inotropic status and the attenuation of the ability to generate contractile force in diabetic hearts has long been associated with defects in contractile proteins and depression in the ATPase activities of contractile proteins. The ATPase activity of cardiac contractile proteins is known to directly influence measurements of heart contractility (+dP/dt). Thus, depressed myocardial contractility in the diabetic heart has been shown to reflect reduced myosin, actomyosin, and myofibrillar ATPase activities (135,215,355-358). The ATPase activity of cardiac myosin is closely associated with the shortening speed of the muscle. Just after one week of diabetes, the myosin ATPases activity has been shown to decrease (135). The decline in myosin ATPase activity is also associated with a change in myosin isoenzyme distribution such that there becomes a predominance of the slow (V_3) isoform of myosin (135). We also observed a redistribution of myosin isoenzymes in diabetic hearts at 2 weeks, followed by a later fall in myofibrillar ATPase activity and subsequent depression in +dP/dt. Our results with respect to

myofibrillar contractile function, myosin isoenzyme shifts (V_1 <-> V_3) and +dP/dt are in general agreement with other studies and these depressions were all reversed by insulin treatment.

In this study, it was observed that the SR Ca^{2+}-uptake was significantly depressed in hearts from chronically diabetic rats, in addition to the SR Ca^{2+}-stimulated ATPase and Mg^{2+}-ATPase activities. These alterations occurred by 10 days after alloxan administration. Depressions in these SR activities have been reported by numerous investigators utilizing alloxan- and STZ-induced diabetic rats. Defects in cardiac relaxation (-dP/dt) have been attributed to depression in the SR Ca^{2+} uptake (123,370,372). The -dP/dt is considered by most to be a functional correlate of the cardiac SR because the rate of Ca^{2+} uptake by the cardiac SR primarily influences this functional parameter. Our observation of depressed SR Ca^{2+} uptake and concomitant depression of -dP/dt is consistent with the role of the SR as a primary subcellular determinant of cardiac relaxation rate. The SR has been considered to be a major factor in the relaxation process by actively accumulating Ca^{2+} from the cytoplasm and existing essentially as a Ca^{2+} storage site. Furthermore, the cardiac SR is known to be regulated by a number of factors including calmodulin (532), cAMP dependent protein kinase (533), and monovalent cations, particularly K^+ (534). The data presented by Lopaschuk et al (375) showed that these regulations did not play a major role in the depression of cardiac SR Ca^{2+}-transport observed in STZ-induced diabetes in the rat. In our preliminary studies, we examined (n=2) the effect of these regulators (data not shown) in various preparations and also found no difference between the stimulation in controls vs. diabetics, at earlier time points compared to that by

Lopaschuk et al (375).

In our studies, we report as with many others, a reversal of -dP/dt along with a reversal in the depressed SR Ca^{2+}-stimulated ATPase activity and SR-Ca^{2+} uptake activity. Although a direct action of insulin on SR Ca^{2+} transport has been reported (373), it is not clear whether this effect actually plays a role in the defects observed in the insulin-deficient diabetic state. It has been suggested that the hypothyroid state of the diabetic animals could be responsible for the defect in SR function. In this regard, it should be noted that hypothyroidism has been shown to cause a depression in cardiac SR Ca^{2+} transport (374). However, treatment of diabetic animals with thyroxine to restore circulating thyroid hormone levels did not abolish the defect in cardiac SR Ca^{2+} uptake activity (123). Thus it was concluded that the hypothyroid state of the diabetic animals was not responsible for the lesion. As discussed previously in the literature review (123), lipid accumulation can affect SR Ca^{2+}-uptake in diabetes, but insulin reversal of the high lipid state did not reverse the impaired cardiac function.

Our model and the severity of diabetes may play a role in the observed depression of the cardiac SR function. It is possible that the severe, ketotic (but not lethal) state may, perhaps influence the early depression. This ketotic state may result in an advanced and progressive decline in membrane fluidity and enhanced lesions in the SR and accumulation of lipid droplets. An early decline in SR Ca ATPase activity (1 week after STZ administration) has been reported also by Katagiri et al (380a) in contrast to the much later decline (4 weeks post STZ and alloxan injection) reported by others. Katagiri et al (380a)

reported a significant depression in the SR Ca^{2+}-dependent ATPase at 1 week; SR Ca^{2+}-dependent ATPase activity decreased markedly to about 40% of that of the control rat. They also reported a further decrease until 16 weeks. In the present study, a 21% decrease in Ca^{2+}-stimulated ATPase activity of the SR was evident 10 days after alloxan administration. By 12 weeks, SR Ca^{2+}-stimulated ATPase activity had declined 40% in comparison to control SR Ca^{2+}-stimulated ATPase activity. Katagiri et al (380a) did not examine ATP-dependent Ca^{2+} uptake; however, an increase in the 100,000 Dalton protein band corresponding to the major SR Ca^{2+}-ATPase protein was observed along with total phospholipid content of SR. It was suggested that the increase in SR membrane components might be an attempt to compensate for the impaired SR function. Overall, the results presented in the study by Katagiri et al suggests a sequential process in the pathophysiological alterations in the diabetic myocardium. Impairment of Ca^{2+}-ATPase was noticed earliest, i.e. within one week after the initiation of diabetes, and then increases in the proteins suggesting the major ATPase and phospholipid content followed in the relatively chronic period. At the same time, activity of Ca^{2+}-dependent actomyosin-ATPase activity significantly diminished without detectable compositional changes in electrophoresis. The alterations in the diabetic myocardium in that study, including SR Ca^{2+} ATPase activity were normalized by daily treatment with insulin, as reported by us and many others.

Changes in cardiac calcium metabolism may also underlie impaired contractile functioning. Because removal of calcium from myocardial cytoplasm is believed to facilitate cardiac relaxation, it is also possible that depression of calcium transport not only by the SR, but

also by the sarcolemma could lead to impaired cardiac relaxation. In this study, we examined the changes in various sarcolemma transport proteins involved with calcium fluxes (Na^+-Ca^{2+} exchange, Ca^{2+}-pump) and the activity of the Na^+-pump (expressed as Na^+-K^+ ATPase activity) in the rat model of alloxan diabetes. The results showed that there were significant depressions of the Na^+-pump and Ca^{2+}-pump (Ca^{2+}-stimulated ATPase) as early as 10 days after alloxan administration. In addition, ATP-dependent Ca^{2+} uptake and Na^+-Ca^{2+} exchange activities were also significantly depressed by 14 days. Only sarcolemmal Mg^{2+}-dependent ATPase activity remained unchanged throughout the course of this study; this finding is also in agreement with the study of Makino et al (457), utilizing STZ-induced diabetic rats. The results presented here with respect to depression in sarcolemmal calcium handling and depressed Na^+-pump activity have been reported consistently utilizing the STZ-diabetic rat model (457) and with respect to Na^+-pump in alloxan diabetic dogs (455). The detailed time course study by Makino et al (457) indicated that changes in the sarcolemma with respect to Na^+-Ca^{2+} exchange, and Ca^{2+}-pump activity occurred 4 weeks after STZ administration. Other studies have only examined Na^+ pump activity and Ca^{2+} pump activity at 6-8 weeks after STZ-administration.

Our overall results with respect to sarcolemma, SR and myofibrillar functions in alloxan-diabetic rats demonstrate clearly that membrane dysfunction is an early occurrence, with dysfunction occurring later in the contractile proteins. Various studies have shown that pathological changes in the myocardium begin to occur at 4 weeks and become maximal at 8 weeks after the induction of diabetes. Our studies show that membrane

defects involving SR and SL occur concurrently as early as 10 days, leading to impairment of cardiac function. No mechanism at this point can account for this early depression, although Katagiri et al (380a) has also observed early depressions (1 week past STZ administration) in SR function. It should be pointed out that our sarcolemmal and SR yields were not significantly different across various treatment groups, and the data reported in this study indicate minimal, yet equal contamination across the various membrane preparations. Thus, differential contamination was not a confounding factor. While no clear reason can be given for our early observed depression in sarcolemmal and SR functions, it should be stated that many factors could account for our findings. These may be: (i) Severity of diabetes, (ii) ketotic nature of alloxan diabetes, (iii) early, progressive ultrastructural damage to the various subcellular organelles, or (iv) early derangement in the microcirculation and enhanced microvascular transport between capillary and myocytes, (v) membrane lipid alterations. It has been observed that thyroid hormone status can influence subcellular and biochemical activity, as well as sarcolemmal Ca^{2+} channels and Ca^{2+} transporter. In addition, whether this alloxan model causes early, aggressive changes in Ca^{2+}-handling, Na^{+}-handling or K^{+} fluxes at an electrophysiological level remains to be seen, as no comparative study exists to date between STZ- and alloxan model in these respects. Changes in thyroid hormone levels, which can have dramatic effects on electrical and mechanical activity also occur within only a few days in both the STZ and alloxan model. It would therefore be important to examine closely the degree of depression in both models, with comparative severity of diabetes.

C. Alloxan-Diabetic Rabbit Model: Subcellular Changes and General Characteristics

The induced-diabetic (hyperglycemic-glycosuric) rabbit, as a model of human diabetes, has been produced by reduction in pancreas mass and injury to pancreatic beta cells (535). Since their first report of hyperglycemia induced by alloxan in the rabbit, a variety of dosages and methods of administration have been utilized by several investigators to achieve a permanent state of diabetes in the rabbit (536). These investigators (536) report there is extreme variability in individual rabbits susceptibility to the diabetogenic effects of alloxan. In the present study a single dose of 125 mg/kg alloxan in the rabbits resulted in a stable, long-term diabetic state characterized by elevated plasma cholesterol and glucose levels, and reduced plasma insulin levels. Our precautionary measures (outlined in methods) resulted in a low (<10%) mortality rate. Our study is in agreement with others (136,137,234,320,358,537) with respect to changes in body weights, heart weights, as well as plasma alterations of glucose, insulin and cholesterol. Bhimji et al (136) reported that hyperglycemia alone did not significantly affect body weight of the diabetic animals. However, hyperglycemia in the presence of hyperlipidemia significantly decreased not only the body weight, but also the heart and left ventricular weights of the diabetic animals. Bhimji et al (234) also reported significant myocardial morphological damage in alloxan-induced diabetic rabbits. They observed myofibrillar disarrangement, mitochondrial damage, increased lipid droplets and glycogen granules, and dilated SR that contained varying degrees of electron-dense material. All of these ultrastructural alterations were quite evident by 10 weeks after alloxan

administration.

Our results of a depression of myofibrillar Ca^{2+}-stimulated ATPase and SR Ca^{2+} and Mg^{2+}-stimulated ATPase are consistent with the previously described hemodynamic correlates reported for this study with alloxan-diabetic rats and is in agreement with the study by Bhimji et al (136), using alloxan diabetic rabbits. Bhimji et al (136) reported that hemodynamic parameters in anesthetized rabbits such as: left ventricular pressure, +dP/dt, and heart rate were significantly depressed in diabetic rabbits at 10 weeks. In addition, in agreement with the present study, depressions in SR-ATPase activity were observed whereas sarcolemmal Na^{+}-K^{+} ATPase was unchanged. However, Bhimji et al (136) reported a significant depression in myofibrillar Mg^{2+}-ATPase activity whereas, we did not observe any significant change; no reason at this time can be advanced for this observation.

In the study by Pollack et al (358), they reported no statistically significant difference in actin-activated Mg^{2+}-ATPase activity. It is thought that small differences between experimental animals and heterogenous control groups might be missed at the low level of activities of this enzyme. Ca^{2+}-stimulated ATPase activity of the myofibrils was significantly depressed in the above study (358). Although we did not examine V_1, V_2, V_3 composition in our rabbits, Pollack et al (358) reported significant elevations (90-95% V_3) in the percentage of V_3 in diabetic rabbit heart at 1, 3 and 6 mo after alloxan administration. Our reported biochemical studies in myofibrils, SR and depression in the Na^{+}-Ca^{2+} exchange are in congruency with not only the observed in vivo hemodynamic changes reported by Bhimji et al (136), but are in accord with the papillary muscle experiments conducted

by Fein et al (320) in the alloxan model of diabetes. In that study, they demonstrated marked differences in myocardial mechanics between chronically diabetic rabbits and their normoglycemic controls. These changes included prolonged time to peak tension and one-half relaxation isometric contraction and prolonged time to peak shortening and diminished shortening velocity in isotonic contraction. Although the rate of contraction was slowed, duration of contraction was increased so that developed tension and peak shortening were generally unaltered in comparison with controls. The results of our studies with diabetic rabbits illustrate a common depression in myofibrillar Ca^{2+} ATPase and in SR-Ca^{2+} ATPase in diabetic rabbits as with diabetic rats. While we only examined subcellular activities at ~10-12 weeks, it is unclear in the rabbit (as we reported with the rat) which of the subcellular organelles display the earliest biochemical defect. The exact molecular mechanism(s) responsible for the regulation of the Na^{+}-Ca^{2+} exchanger in normal rats and rabbits as well as in pathological states, such as diabetes is not known. Since the activity of the Na^{+}-Ca^{2+} exchanger was depressed in both diabetic rats and rabbits, unlike the Ca^{2+} pump, it is thus possible that the Na^{+}-Ca^{2+} exchanger activity is the most sensitive Ca^{2+} transport protein to either hypoinsulinemic, hyperglycemic, hyperlipidemic (or any combination of these) manifestations of the diabetic state.

It is also of note to point out that the thyroid hormone levels of the diabetic rabbits in the above studies (136,358), unlike diabetic rats were not significantly altered with respect to their age-matched controls. Thus, since thyroid hormone status has been shown to be important for myocardial function and cardiac subcellular enzymatic

activities, this difference between rats and rabbits with respect to thyroid status in diabetes may not be important for Na^+-Ca^{2+} exchange activity in the diabetic rabbit heart sarcolemma, future studies should examine in more detail Na Ca exchange regulation. In addition, other determinants of Ca^{2+} transport (i.e. Ca^{2+} channels) in relation to the Na^+-Ca^{2+} exchanger need further study. At this time, an explanation for lack of depression in the Ca^{2+}- and Na^+-pump in the diabetic rabbit heart sarcolemma awaits further study. Taken together, however, our studies suggest a lack of Ca^{2+}-handling (either Ca^{2+} overload/impairment in Ca^{2+} handling) in both the alloxan diabetic rat and rabbit.

D. Molecular Changes in the mRNA Transcripts of: Alpha-Myosin Heavy Chain (A-MHC), SERCA2 and Alpha$_1$-Subunit of the Na^+-K^+ ATPase

1. Alpha-myosin heavy chain

The existence of three myosin isoforms has been well documented in the rat ventricle (364). The myosin V_1, which has a high myosin ATPase activity, is composed of two myosin heavy chain (MHC) alpha polypeptides; myosin V_2, which has an intermediate myosin ATPase activity, consists of a heterodimer containing MHC alpha and MHC beta and myosin V_3, which has a low ATPase activity, contain two MHC beta polypeptides. Diabetes mellitus leads to a marked decrease in myosin ATPase activity, and this decrease in myosin ATPase activity is accompanied by a decreased predominance of myosin V_1 and an increased predominance of myosin V_3 isoform (135,361). We have already shown that a marked depression in V_1 composition after 2 weeks of diabetes in the alloxan diabetic rat followed by depression in the myofibrillar Ca^{2+} ATPase activity. Our study, in agreement with many others have shown that hearts which have

an increased predominance of myosin V_3 isoform and a decreased predominance of myosin V_1 display a decreased velocity of contraction; this well documented in the diabetic heart. In this study, we wanted to determine whether the decrease in myosin isoforms observed in the diabetic heart is accompanied by corresponding changes of the mRNA coding for the MHC alpha. Our results indicated that in diabetic animals, MHC alpha expression declined dramatically by 2 weeks in diabetic hearts. Our results are in agreement with Dillman et al (538) who also showed a depression in the expression alpha MHC transcript. This and our study, therefore, indicate that alterations in myosin is isoenzyme predominance in the diabetic rat heart are mediated by corresponding changes in the level of the mRNA's coding for MHC alpha. It is thought that these alterations might result directly from diminished insulin action. It is also more likely that they are due to secondary changes such as a decrease in glucose composition and an increase in fatty acid oxidation that occur in diabetic hearts (538). Dillman et al (538) showed that chronic alterations in fatty acid and glucose consumption appears to influence myosin ATPase activity, myosin isoenzyme predominance, and MHC alpha mRNA levels.

It is not clear why at 5 weeks and 6 weeks in our study that the alpha-MHC expression increases back to control levels, whereas myofibrillar Ca^{2+}-stimulated ATPase activity reported in our study is quite depressed. It is not clear what the molecular signal(s) are for this change over the duration of diabetes in the alloxan rat model. It is known that hypothyroidism can depress the expression of the alpha MHC (539). Thyroid hormone levels have been shown to be depressed in diabetes in both the STZ- and the alloxan-induced model of diabetes. It

is not clear presently what compensatory mechanisms may be involved in the observed biphasic expression of the alpha MHC transcript in diabetes mellitus in the rat at this time point.

2. SERCA2

Several reports including this present study have indicated that handling of Ca^{2+} by cardiac myocytes is altered in diabetes (123,322,372,380a). The SR is known to be the major intracellular organelle that sequesters intracellular Ca^{2+} and regulates the relaxation and the tension development of the myocardium. Calcium uptake by the SR in cardiac myocytes is driven by the Ca^{2+} ATPase (SERCA2) (540). The cardiac SERCA2 gene encodes two alternatively spliced transcripts, one expressed in cardiac and slow-twitch muscle (SERCA2a), and the other expressed in smooth muscle and non-muscle tissues (SERCA2b) (541-545). In the cardiac myocytes only the SERCA2a isoform is expressed during development, neonatal and adult hearts, as well as in various physiological and pathological forms of cardiac hypertrophy and heart failure (546). Several investigators have provided evidence that the Ca^{2+} transport function of SR is altered in myocardial hypertrophy in experimental animals as well as in humans (546). Such alterations in SR function are considered primarily to be due to changes in the expression of mRNAs encoding SR Ca^{2+} transport proteins.

As we and others have shown, insulin-dependent diabetes invariably results in heart dysfunction characterized by decreased velocity ofcontraction, prolonged diastolic relaxation time, decreased cardiac output and high filling pressures (547). Chronic diabetes due to streptozotocin or alloxan administration rats has been shown to date to induce cardiomyopathy characterized by defects in cardiac function,

ultrastructure and metabolism similar to those observed in diabetes mellitus (123,356). Studies from different laboratories including the present study have shown that prolonged relaxation time of the diabetic myocardium is correlated with decreased SR Ca^{2+} uptake and Ca^{2+} ATPase (Ca^{2+}-stimulated ATPase) activities (123,370,372); however, the molecular mechanisms for these changes are poorly understood. Therefore, one of the objectives in this study was to determine whether alterations in SR Ca^{2+}-pumping activities in the alloxan-diabetic rat heart was associated with a decrease in the SERCA2 mRNA level. Our results showed no significant alteration in SERCA2 gene expression at 2 weeks after alloxan administration but 3 and 5 weeks after alloxan administration, SERCA2 was significantly (+30%) elevated with respect to control values. At a later time point, such as 6 weeks after alloxan injection, no significant change in expression was observed.

Results reported for changes in SERCA2 mRNA levels in the STZ diabetic rat heart have been controversial. Zarain-Herzberg et al (548) reported that Ca^{2+} uptake and Ca^{2+}-stimulated ATPase activities were depressed 3 and 5 weeks after STZ administration. In addition, using Northern analysis and slot blot techniques, they observed a small (less than 20%), but not statistically significant, decrease in the relative level of expression of SERCA2 mRNA (normalized with 28S rRNA) in 3 or 5 weeks diabetic left ventricles compared to age-matched control rats. While insulin treatment restored both Ca^{2+} uptake and Ca^{2+}-stimulated ATPase activities, insulin treatment of diabetic animals for 2 weeks did not significantly alter the SERCA2 mRNA level in the left ventricular myocardium compared to the age-matched control or diabetic animals. Similar results were reported from slot blot analysis (548). Dillmann

(538) also reported no change in cardiac SERCA2 mRNA levels in hearts of rats 4 weeks after STZ administration. In contrast to these two above studies, one study reported a 50% reduction in SERCA2 mRNA level in rat hearts after 4 weeks of streptozotocin injection (538). Our study, reporting a 30% increase in SERCA2 expression at 3 and 5 weeks is at odds with the latter and three studies. The highly significant decrease in SERCA2 mRNA reported in the previous study (549) and the small decrease reported in Zarain-Herzberg et al (548) has been suggested perhaps, as a consequence of a decrease in the circulating levels of thyroid hormone in the STZ-induced diabetic rat (123). It has been shown that in 4 week propylthyouracil-induced hypothyroid rabbits, the level of SERCA2 mRNA in the heart is decreased approximately by 35% compared to euthyroid animals (550). SR Ca^{2+} ATPase mRNA levels are also markedly decreased in hypothyroid rat hearts also (551). It is of special interest to be reminded at this point of the work by Katagiri et al (380a). They showed an early depression of the SR Ca^{2+} ATPase activity but an increase in the Ca^{2+} ATPase (+150% vs. control level) protein at 4 through 16 weeks after STZ administration. Our reported increase in SERCA2 mRNA between 3 and 5 weeks after alloxan administration and the findings of the previous study would seem consistent in support of the notion that there may be an attempt to compensate for impaired SR function. Nevertheless, alternative explanations for the SERCA2 (Ca^{2+}-stimulated ATPase) activity defect in the diabetic myocardium include a generalized alteration in phospholipid composition as well as changes in the phosphatidylethanolamine N-methylation in the SR membranes (123,552).

3. Alpha$_1$-subunit of the Na^+-K^+ ATPase

The sodium-potassium ATPase (Na^+-K^+ ATPase or Na^+-K^+-pump)

enzyme is a plasma membrane-bound, oligomeric protein consisting of an alpha- and beta-subunit in a one-to-one stoichiometry (553). The alpha-subunit is well recognized as mediating the catalytic processes of the enzyme whereas a clear understanding of the function of the beta-subunit remains more elusive. Nevertheless, the accumulating evidence appears to support the hypothesis that the beta-subunit facilitates the correct assembly and transport of the alpha-subunit into the plasma membranes (554,555). The Na^+-K^+ ATPase is now known to belong to a multigene family. Three distinct isoforms of the alpha-subunit ($alpha_1$, $alpha_2$ and $alpha_3$) have been identified by molecular, genetic and immunological techniques (556,557). Similarly, three isoforms for the beta-subunit ($beta_1$ and $beta_2$) have been identified (556). The alpha and beta isoform genes are expressed in a tissue and cell-specific manner and are subject to developmental and hormonal regulatory influences (556,557). In rat cardiac tissue, the Na^+-K^+ ATPase alpha isoform genes exhibit a complex pattern of expression during development (58). The $alpha_1$ mRNA is the major alpha isoform transcript (~70-75% of total alpha mRNA abundance) expressed at all developmental stages (i.e. fetal to adult). In the human heart, the $alpha_1$ mRNA isoform is also the major alpha isoform (~63%).

Changes in thyroid status have been well documented to affect the sensitivity of the heart to cardiac glycosides. At the cellular level, thyroid hormone has been shown to increase the Na^+-K^+ ATPase activity and number of Na^+-K^+ ATPase sites in various types of tissue, including heart cells (558-561). In the hypothyroid state, the $alpha_1$ isoform mRNA is the predominate form, whereas administration of T3 induces primarily the $alpha_2$ isoform of mRNA (562,563). Since diabetes

is associated with depressed thyroid and insulin levels, we examined whether there are any changes in the expression of the $alpha_1$ subunit of the Na^+-K^+ ATPase in alloxan-induced diabetic rat ventricles. Our studies show a significantly depressed expression of the $alpha_1$ subunit by 6 weeks after alloxan administration. However, since functional activity of the heart and biochemical activity of the Na^+-pump (measured as Na^+-K^+ ATPase activity) were depressed as early as 10-14 days, we cannot conclude that there is a direct association between early $alpha_1$ isoform mRNA levels and Na^+-K^+ ATPase activity. Since diabetes is associated with a number of metabolic disorders, including impaired protein synthesis, and increased protein degradation, and depressed insulin levels; these factors in addition to early membrane changes are more likely to influence Na^+-K^+ ATPase activity. In human ventricles from failing hearts, the expression of $alpha_3$ isoform has been shown to be elevated (564). Conversely, these studies have shown heart tissue specimens from failing ventricle exhibited a decreased expression of $alpha_2$. In both normal and diseased hearts, isoforms tended to move in parallel (564).

Zahler et al (564) reviewed studies in which several groups have demonstrated decreases in the $alpha_2$ and $alpha_3$ isoforms in hypertrophied animal hearts. They also commented that cardiomyopathic hamsters also had a 33% reduction in cardiac Na^+-K^+ ATPase concentration. In our study, we did not quantify the Na^+-K^+ ATPase concentration and therefore, reduced numbers at a later stage of diabetes (6 weeks or longer) may be evident. Since it is not clear whether isoform mRNA levels may be proportional to amounts of pump isoform proteins or be proportional to numbers of functional pump units.

Further work will be needed to examine both the molecular and biochemical regulation of this enzyme in diabetes. Our reported finding (data not shown) of no difference between the expression of the sodium-calcium exchanger in diabetes vs. control hearts, is not unsurprising. Membrane factors, phosphorylation status, lipid alterations and metabolic state, and involvement of the sarcoplasmic reticulum may likely be more important regulators of the Na^{+}-Ca^{2+} exchange than molecular transcriptional regulation of its function.

VI. CONCLUSIONS

A. Alloxan-induced diabetic rats displayed elevated plasma glucose concentrations and depressed plasma insulin levels, consistent with a Type I, poorly controlled, chronic diabetic state. Depressions in body weight and ventricular weight resulted in an elevated ventricle-to-body weight ratio. These general manifestations of the experimental diabetic rats were all normalized by chronic insulin administration for 4 weeks.

B. Assessment of hemodynamic parameters revealed only an impairment in the relaxation index, -dP/dt, 2 weeks after alloxan administration. In agreement with this diastolic change, a pronounced depression in both sarcoplasmic reticulum (SR) uptake and SR Ca^{2+} ATPase activities were noted at 10 days after alloxan administration. These parameters were subsequently normalized 4 weeks after chronic insulin administration.

C. Hemodynamic parameters, such as heart rate, +dP/dt, LVSP and MAP were depressed while LVEDP was increased in 4 week diabetic rats in comparison to control rats. Contractile protein function (measured as myofibrillar Ca^{2+}- and Mg^{2+} ATPase activities) was also decreased concurrently along with the depressed systolic function (+dP/dt, LVSP). These parameters were also normalized after 4 weeks of insulin treatment.

D. A pronounced change in myosin V_1 and V_3 composition was noted 2 weeks after alloxan administration in the diabetic rats. V_1 decreased and V_3 increased to abnormal proportions. Insulin administration normalized the V_1 and V_3 proportions.

E. Sarcolemmal (SL) enzyme systems involved in normal intramyocyte

Na^+ and Ca^{2+} concentrations, such as the Na^+-K^+ ATPase and the SL Ca^{2+}-pump (Ca^{2+}-stimulated ATPase) were depressed concurrently at 10 days, as seen for the SR Ca^{2+} pump. A later depression in both Na^+-Ca^{2+} exchange and ATP-dependent Ca^{2+}-uptake activities were noted in 2 weeks diabetic rats. These observations indicate that membrane dysfunction with respect to Ca^{2+} uptake and Ca^{2+}-extrusion are impaired prior to contractile protein dysfunction and are implicated in early, impaired diastolic function in alloxan-induced diabetic rats.

F. Rabbits made diabetic with alloxan displayed reduced body and heart weights and had elevated plasma glucose and cholesterol and depressed plasma insulin concentrations. Diabetic rabbits at 12 weeks after alloxan administration showed marked reductions in SR Ca^{2+}-pump (SR Ca^{2+} ATPase) and myofibrillar Ca^{2+} ATPase activities. Only the Na^+-Ca^{2+} exchanger of the SL was markedly reduced; there were no changes in the Na^+- nor Ca^{2+}-pump activities in alloxan-diabetic rabbits. These results show that subcellular abnormalities in diabetes are not limited to the rat model.

G. Molecular biological studies undertaken revealed depressions in the alpha myosin heavy chain expression as early in 2- and 3-week diabetic rats; no significant change with respect to control was observed after 3 weeks. SERCA2 expression was only significantly increased in 3 and 5 weeks diabetic rats, whereas, the expression of the Na^+-Ca^{2+} exchange mRNA remained unchanged at 2 and 3 weeks (only time points examined, data not shown). The expression of the $alpha_1$-subunit of the Na^+-K^+ ATPase was significantly

reduced in 6 weeks diabetic rats. These molecular studies indicate only an early positive correlation between alpha MHC expression and early myosin isoenzyme changes with subsequent depression in myofibrillar Ca^{2+}-ATPase activity. It appears that factors, other than transcriptional changes affect the activity of the SR, Na^{+}-K^{+} ATPase and the Na^{+}-Ca^{2+} exchanger in alloxan-induced, chronic diabetes in rats.

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