THE EFFECT OF EXPERIMENTAL DIABETES ON DRUG INDUCED

RESPONSES IN CARDIAC TISSUES OF THE RAT

by

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ABSTRACT

The effect of experimental diabetes mellitus on the response
of isolated cardiac tissues to the β adrenergic agonist d,l isoproterenol
and the cardiac glycoside ouabain was examined. The relationship
between the duration of chemically induced diabetes and the response
to these drugs was also investigated.

Basal developed tension was not different in control vs. diabetic
papillary muscles 7 days or 70 days after the induction of diabetes.
Tissues from 7 day diabetic animals responded to d,l isoproterenol in a
similar manner to tissues from control animals at each drug dose. There was
a non-significant depression in the response of both papillary muscles and
left atria from 70 day diabetic rats. This trend was evident throughout
the dose-response curves.

The basal rate of spontaneously beating isolated right atria
from 7 day STZ diabetic rats was significantly depressed compared to
control, while that of alloxan diabetic animals was depressed to a
smaller degree. There was no difference in the maximum response of
these tissues to d,l isoproterenol. The basal rate was not different
in atria from 70 day diabetic animals as compared to controls. Tissues
from 70 day diabetic rats demonstrated a diminished response to d,l
isoproterenol throughout the dose response curve however this
depression was not statistically significant.

There was no difference in tension development in left atria or
papillary muscles at any time point. Seven days after the induction
of diabetes both atria and papillary muscles demonstrated a non-
significant depression of the ouabain dose response curve. Papillary

muscles from 70 day diabetic animals displayed a significant depression in these dose response curves at ouabain concentrations greater than 10^{-5} M. Atria from six month diabetic rats demonstrated significantly depressed curves at concentrations greater than 3×10^{-5} M.

Ouabain produces a monophasic dose response curve in left atria and a biphasic dose response curve in papillary muscles. Catecholamine release does not appear to be involved in these responses.

Chronic alloxan and streptozotocin diabetes produces changes in the myocardium of rats characterized by a diminished inotropic response to the cardiac glycoside ouabain. This depression is not accompanied by a statistically significant decline in the maximum inotropic or chronotropic response to isoproterenol.

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LIST OF ABBREVIATIONS

STZ   streptozotocin

ED_{50} dose of agonist which produces 50% of maximum response

I_{50} dose of antagonist which inhibits 50% of maximum response

(+)dP/dt   the maximum rate of pressure development

-dP/dt   the maximum rate of pressure decline

CK   Chenoweth Koelle

LVEDV   left ventricular end diastolic volumes

cAMP   cyclic adenosine mono phosphate

cGMP   cyclic guanosine mono phosphate

SR   sarcoplasmic reticulum

CoA   coenzyme A

RIA   radioimmunoassay

tim   timolol maleate

iso   d,l isoproterenol

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INTRODUCTION

I Diabetes Mellitus and Diabetic Cardiomyopathy

1. Introduction

Diabetes mellitus is the name given to a group of disorders
characterized by an excess of glucose in the plasma resulting from
a lack of insulin or a lack of insulin activity. This disease has
afflicted individuals for thousands of years. A half century ago the
discovery of insulin by Banting and Best was heralded as a cure for diabetes
(Banting and Best, 1922 and Banting et al., 1922). Its therapeutic use has
prevented the death of vast numbers of diabetics and as the life expectancy
of this population has increased, the role of the clinician has evolved.
Between 1897 and 1914 sixty four percent of diabetics at the
Joslin Clinic died in a diabetic coma while cardiovascular
disease claimed only eighteen percent. Today seventy-five percent
die from cardiovascular complications and only one percent die in a
diabetic coma (Marble, 1972). Diabetics are prone to neuro-
pathy, retinopathy, renal failure, congestive heart failure, stroke
and coronary heart disease (West, 1978a). In the past, the
high risk of cardiovascular disease was attributed to the promotion
of atherosclerosis by diabetes (Boucher et al., 1979). The Framingham
study (Kannel et al, 1974) reported that congestive heart failure in
diabetics could not be attributed to atherosclerosis and coronary heart
disease and that some form of cardiomyopathy must be associated with
diabetes mellitus. Ledet et al, 1979 have concluded that the cardio-
vascular complications of diabetes cannot be fully accounted for by
atherosclerosis. In light of these observations and in an attempt
to provide improved health care, a number of studies have attempted

to define changes in the myocardium of diabetics.

The information available concerning cardiovascular disease in diabetics includes the results of epidemiological studies, reports of cardiovascular function in human diabetics and the results of studies involving diabetic animals. The profound effect of diabetes on life expectancy has evoked considerable interest in the epidemiology of the disease from scientists, clinicians and life insurance companies. One of the most useful surveys has been the Framingham Study (Kannel et al., 1974). Initiated in 1949 to explore cardiovascular disease in 5209 men and women aged 30-62 years, this study has provided valuable information regarding the role of diabetes as an independent risk factor. Individual case studies are of limited value in terms of defining changes in the myocardium of the diabetic population, however, some reports concerning myocardial function in groups of diabetic patients are available (Regan et al., 1977).

The development of diabetes is influenced by a number of factors including adiposity, genetics, viral infections, diet, race, exercise, sex and parturition (West, 1978b). A number of these factors also influence the development of cardiovascular diseases. It has been difficult, in epidemiological studies, to assess the independence or co-dependence of these risk factors in the presentation of diabetes mellitus and cardiovascular disease. The use of animal models of diabetes has allowed control of some of these factors and has allowed researchers to observe large numbers of subjects. Diabetes occurs spontaneously in many species however the extreme rarity has prompted scientists to induce diabetes in animals by various means in order to study the disease (Mordes and Rossini, 1981). Pancreatectomy was

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was the first method used to induce diabetes in animals; control animals were sham operated. The major disadvantages of this procedure were the trauma of major surgery and the loss of pancreatic exocrine function.

Recently, selective breeding techniques have led to the isolation of strains of rodents which produce a large number of spontaneously diabetic offspring. Non diabetic littermates serve as controls. The mode of inheritance is more clearly defined in some cases than in others. Insulin levels, relative body weights and occurance of ketosis are dependent on the individual model of spontaneous diabetes. These animals, when available, are very useful however they require a large amount of space and care and therefore expense. (Mordes and Rossini, 1981).

The use of β-cytotoxic drugs has become quite popular. Diabetogens such as alloxan and streptozotocin (STZ) selectively destroy pancreatic β cells (Dunn et al., 1943 and Junad et al., 1967). Both of these drugs produce glucose intolerance in a number of animals including the rat. Guinea pigs are not sensitive to alloxan but will respond to STZ (Rerup, 1970). The use of two different diabetogens should ensure that the observed effects on the cardiovascular system result from diabetes and not from direct toxic actions of these drugs.

Alloxan and STZ produce a diabetic state which is characterized by hyperglycemia and insulin deficiency (Rerup, 1970). The severity of the diabetic state is dependent on the dose of diabetogen used (Rossini et al., 1975 and Ganda et al., 1976).

Insulin injections are sometimes given to control hyperglycemia

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in experimental animals. The relationships between hyperglycemia and the sequelae of diabetis mellitus are not clear. The severity of the diabetic state as well as the presence or absence of exogenously administered insulin are important factors to be considered when analyzing the available literature.

A relationship between the duration of diabetes and the development of heart disease has been suggested (West, 1978a). One would expect that a cardiomyopathy associated with diabetes might not be evident in newly diabetic animals but might appear and worsen with the duration of the disease. It is therefore important, when examining reports of cardiovascular function in diabetic animals, to determine the duration of the diabetic state.

2. Functional and Corresponding Morphological Changes in the Diabetic Myocardium

Regan et al. (1977) observed that hearts of human diabetics were unusually stiff as evidenced by an elevation in left ventricular end diastolic pressure and a reduction in end diastolic volume. A similar observation was made in the hearts of dogs made chronically diabetic with alloxan (Regan et al., 1974). The diabetic heart also appears to have a longer pre-ejection period, a shorter left ventricular ejection time, a higher ratio of pre-ejection period to left ventricular ejection time (Ahmed et al., 1975) as well as a prolonged isovolumic relaxation time (Ahmed et al., 1975 and Rubler et al., 1978). Recently, Regan et al. (1981) compared the function of hearts of one year alloxan diabetic dogs with those of non diabetic dogs. One half of the diabetic animals were maintained throughout the study on daily

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insulin injections. Animals were anaesthetized and hearts were studied in vivo. Basal left ventricular function and contractility were not different in diabetics as compared to controls. During intraventricular infusion with saline, end diastolic pressure reached higher levels in both diabetic groups than in control animals. Although basal left ventricular end diastolic volumes (LVEDV) were not different, hearts from diabetic dogs displayed smaller LVEDV in response to the saline infusion. This would support Regan's earlier observation of increased stiffness in diabetic hearts (Regan et al., 1977). These hearts were not hypertrophied and electron microscopy did not reveal any abnormalities in subcellular organelles. Hearts from both diabetic groups displayed significant elevations of collagen which the authors suggested might account for the diminished compliance. It was noted that control of hyperglycemia by insulin was not sufficient to prevent cardiovascular changes resulting from chronic diabetes mellitus.

Baandrup et al. (1981) reported that the relative amount of connective tissue in hearts of poorly controlled, 9 month STZ diabetic rats was significantly greater than in well controlled or non-diabetic rats. The progressive nature of myocardial abnormalities is emphasized by the fact that Modrak (1980) could not detect any changes in collagen concentration or synthesis in the hearts of rats 3, 6, 18 or 26 weeks after the induction of STZ diabetes. Collagen concentration did increase in both control and diabetic hearts at 18 and 26 weeks however this was probably an age related change.

Haider et al. (1981) investigated the effect of an atherogenic diet (high in saturated fat and cholesterol) on the myocardium of

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eighteen month alloxan diabetic rhesus monkeys. Animals were anaesthetized and heart function was monitored in vivo. Intraventricular saline injections producing increases in preload resulted in decreased stroke work in both diabetic groups (control diet and atherogenic diet) as compared to non-diabetic groups. As had been previously reported in dogs (Regan et al.,1981) left ventricular end diastolic pressure was increased more in diabetics while left ventricular end diastolic volume increased to a lesser extent. Again these observations support the theory of increased stiffness in the diabetic myocardium. While soluble collagen decreased, insoluble-collagen increased in diabetics and might contribute to the wall stiffness. Occlusive lesions were not detected in transmural coronary arteries in any of the groups. The atherogenic diet alone did not appear to produce any great effect on cardiac performance.

Fein et al. (1980) examined left ventricular papillary muscles from streptozotocin (STZ) diabetic rats 5; 10 and 30 weeks after the induction of diabetes. Isometric studies demonstrated that diabetic tissues had a decreased ability to relax; the time to one half relaxation was prolonged and the maximum rate of tension decline was decreased. There were no differences in the passive or active length-tension curves of diabetics and controls. Isotonic studies revealed that the peak velocities of shortening and relaxation were lower in diabetic tissues. From force velocity curves it was evident that diabetics displayed depressed shortening velocities over a wide range of loads. The mechanical changes were not significantly altered by the duration of diabetes (5, 10 or 30 weeks).

Vadlamudi et al. (1982) also investigated the time course of

development of functional changes in diabetic rat hearts. The
function was assessed by measuring the maximum rates of pressure
increase (+dP/dt) and decrease (-dP/dt) in response to changes in
atrial filling pressure in isolated perfused working hearts. Hearts
from 7 day alloxan diabetic rats did not respond differently from
those of control animals however 30, 100 and 240 days after the induction
of diabetes these hearts responded to high filling pressures with
depressed positive and negative DP/dt. Depressed cardiac performance
at high atrial filling pressures was observed in hearts from STZ
diabetic rats 100, 180 and 360 days after treatment. The performance
of hearts from 7 and 30 day STZ diabetic rats was not different from
control. The authors suggested that cardiac functional alterations
may appear in diabetic rats about 30 days after the induction of the
disease.

Miller (1979) reported that isolated perfused working hearts from
3 day alloxan diabetic rats demonstrated a decreased ability to respond
to increased atrial filling pressures. Coronary flow was not affected
however a decrease in aortic output led to a decrease in cardiac
output in hearts from diabetic animals.

The effect of 2, 6, 10 and 28 day insulin therapy on the mechanics
of 6-10 week STZ diabetic rats was also investigated (Fein et al., 1981).
Neither 2 nor 6 day therapy had any effect on the depressed cardiac
muscle performance previously described (Fein et al., 1980), however,
after 10 days there was an improvement and following 28 days of insulin
therapy the mechanical changes were no longer evident. The addition
of insulin to tissue baths did not reverse the mechanical changes in
diabetic tissues.

Penpargkul et al., (1980) reported that 8 weeks after STZ induced diabetes isolated working rat hearts demonstrated diminished cardiac output and stroke work at high filling pressures. Maximum left ventricular pressure and maximum aortic flow rate were not as great in diabetic hearts as in controls. The maximum rate of pressure decline, an index of relaxation, was depressed in hearts from diabetic animals. In agreement with Regan et al. (1981) and Haider et al. (1981), these authors observed a diminished left ventricular end diastolic volume response to increases in pressure. The hearts from diabetic animals were smaller than those from controls and the increase in volume expressed per gram of heart weight was actually greater in diabetic hearts than controls.

3. Autonomic Neuropathy in Diabetes Mellitus

Autonomic neuropathy is commonly encountered in diabetics however its etiology remains a puzzle. Within 3 to 4 days of the induction of diabetes with STZ Kaul and Grewal (1980) observed an increase in sympathetic activity reflected by an increased urinary excretion of catecholamines. Noradrenaline content in diabetic hearts was not different from controls. Twelve day STZ diabetic rats demonstrated significant increases in both serum and ventricular noradrenaline (Paulson and Light, 1981).

Senges et al. (1980) reported that 100 days after the induction of alloxan diabetes in rabbits various changes were evident in automaticity and conduction including lower sinus rate. Small coronary arteries appeared normal however intracellular glycogen accumulation was increased and mitochondria appeared abnormal. Savarese and

Berkowitz (1979) suggested that the bradycardia reported in diabetic animals might be due to a decrease in the number of β adrenergic receptors. These authors reported a 24% decrease in heart rate of 2 month STZ diabetic rats accompanied by a 28% decrease in β receptors in ventricular tissue. The authors did not address the question as to the effect on functioning versus spare β receptors.

Foy and Lucas (1976) observed a decreased sensitivity to iso-proterenol in hearts of 1 week alloxan and 2 week STZ diabetic rats. Vadlamudi and McNeill reported similar inotropic responses to isopro-terenol in hearts from alloxan or STZ diabetic animals. Experiments were performed at various time points between 7 days and 6 months after the administration of diabetogen. The cardiac relaxant effect of isoproterenol (-dP/dt) was depressed in hearts from diabetic animals 7 days, 30 days and 6 months after the induction of diabetes (Vadlamudi and McNeill, 1980, Vadlamudi and McNeill, 1981a and Vadlamudi and McNeill 1981b). These observations suggest that there may be a link between the observed defect in relaxation (Regan et al., 1981) and the autonomic neuropathy.

Miller et al. (1981) investigated the effect of 3 to 4 day alloxan diabetes on the response of isolated perfused rat hearts to epinephrine. Diabetic hearts displayed decreased epinephrine-mediated increases in cAMP and protein kinase activation however the conversion of phosphorylase b to a was increased in diabetic hearts. Propranolol, a β blocker, prevented the epinephrine-induced increase in cAMP and protein kinase activity in control and diabetic animals and while it also blocked the epinephrine induced phosphorylase activation in control hearts it failed to inhibit this conversion in hearts from diabetic animals. The α agonist

phenylephrine had no effect on cAMP or protein kinase activity and activated phosphorylase activity in diabetic but not control hearts indicating the possible involvement of an α receptor. Phosphorylase activation in diabetic hearts was also more sensitive to glucagon, a hormone which does not stimulate α or β receptors. Glucagon-mediated cAMP and protein kinase activation were not different in control as compared to diabetic tissues. The decrease in cAMP and protein kinase activation in diabetic hearts reported in this paper is consistent with the report of a decrease in β receptor number. The increased sensitivity of phosphorylase activation may represent an α receptor involvement or perhaps a direct modification of the phosphorylase.

Ingebretsen et al. (1981) reported that alloxan diabetic rats maintained on insulin for at least 2 weeks and withdrawn from insulin 4 days prior to study displayed no change in basal cAMP, cGMP or protein kinase or phosphorylase activities. Diabetes depressed iso-proterenol induced changes in cAMP and protein kinase activity but had no effect on phosphorylase activation and increased left ventricular pressure. The authors suggested that insulin alters the ability of the heart to accumulate cAMP and interferes with the gain of the amplification cascade system.

Das (1973) reported that one week STZ diabetes had no effect on adenylate cyclase activity of rat hearts however it did result in a depression of cAMP phosphodiesterase activity.

Vadlamudi and McNeill (1982) reported that the time course of isoproterenol mediated cAMP production was not altered by 3 or 30 day

alloxan or STZ induced diabetes. Basal phosphorylase activity was enhanced in both 3 and 30 day diabetic tissues. Isoproterenol induced increases in phosphorylase activation were greater in hearts from diabetic animals at both time points.

Vagal involvement in diabetic autonomic neuropathy has received very little attention however slow gastric emptying and decreased gastric secretory responses to hyperglycemia suggest a possible involvement (Vaisrub, 1978). Functional studies have indicated that, under certain conditions, the diabetic myocardium has a decreased ability to relax (Regan et al., 1981, Vadlamudi and McNeill, 1981a). Cholinergic stimulation produces a negative inotropic response in cardiac tissue and it has been used to investigate myocardial relaxation.

Foy and Lucas (1976) reported a decreased sensitivity to acetylcholine in hearts of 1 to 2 week alloxan and STZ diabetic rats. Vadlamudi and McNeill (1981c) observed no difference in the response to carbachol of isolated perfused working hearts from 7 or 30 day alloxan or STZ diabetic rats as compared to controls. These authors observed a decreased sensitivity to carbachol in hearts from 100 day alloxan or STZ diabetic rats (Vadlamudi and McNeill, 1980) as well as in hearts from one year STZ diabetic animals. These authors also reported an increased sensitivity to this drug in hearts from 6 month STZ and 8 month alloxan diabetic rats (Vadlamudi and McNeill, 1981c).

4. Diabetes and Myocardial Metabolism

Free fatty acid represents approximately 60% of the substrate requirement of healthy hearts (Fig 1). The remaining substrate is

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<u>Fig 1.</u>

The Substrate Supply of the Normal Heart

Glucose, lactate and free fatty acids (FFA) are the major
myocardial fuels accounting for 30%, 10% and 60%, respectively,
of the oxygen uptake in the fasted, basal state.

-from Opie, 1978.

SUBSTRATE SUPPLY
NORMAL HEART
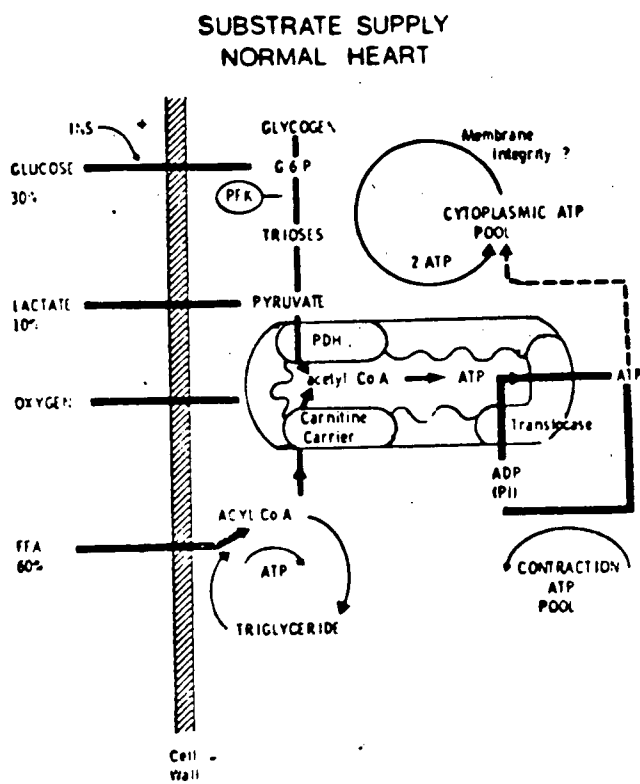
FIG 1

provided by glucose (30%) and lactate (10%) (Opie *et al.* 1971).
Diabetes has a profound effect on the metabolic state of the heart.
The rate limiting step in glucose metabolism is transport across the
plasma membrane. In the absence of insulin this is drastically
reduced resulting in a decrease in intracellular glucose and hence
a decreased contribution as a metabolic fuel. The relative contri-
bution of free fatty acids is increased and this in turn inhibits
several steps in glycolysis including glucose transport (Neely *et al.*,
1969), glucose phosphorylation (Randle *et al.*, 1966) and pyruvate
dehydrogenase (Kerbey *et al.*, 1976). Feuvray *et al.* (1979) suggested
that the enhanced oxidation of endogenous lipids in diabetic hearts
might explain their resistance to insulin stimulation of glucose
transport. The increased fatty acid oxidation in diabetic hearts is
accompanied by altered levels of metabolites. Tissue levels of
triglycerides (Denton and Randle, 1967), long chain acyl CoA,
acetyl CoA, citrate (Randle *et al.*, 1966) and acylcarnitine (Feuvray
et al., 1979) are increased in diabetic hearts. Paulson and Crass (1980)
have demonstrated an increase in triglycerides in the hearts of 12 day
STZ diabetic rats which was prevented by insulin treatment. Murthy
and Shipp (1980) have shown a correlation between heart triglyceride
content and triglyceride synthesis in normal and diabetic rats and
have suggested that the triglyceride accumulation in diabetic hearts
is due, at least in part, to accelerated triglyceride synthesis. It
has also been reported that triglycerides are elevated in papillary
muscles of human diabetics (Alavaikko *et al.*, 1973).

The inhibition of glycolysis in the diabetic myocardium may have

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little effect when the heart is performing at submaximal capacity however the diabetic heart may be less able to withstand conditions, such as increased cardiac work and anoxia, where the heart relies more heavily on the energy produced from glycolysis. Hearse et al. (1975) investigated the ability of isolated perfused working hearts from 6 to 9 day STZ diabetic rats to survive and recover from a 30 minute period of anoxia. Hearts from non diabetic animals recovered very well however hearts from diabetic animals displayed an initial rapid recovery phase followed by a period of cardiac failure and a second, less effective, period of recovery.

Feuvray et al. (1979) reported that hearts from 2 day alloxan diabetic rats responded to low levels of cardiac work in a similar manner to hearts from non diabetic rats when mechanical function was determined on isolated perfused working hearts. Hearts from both groups of animals recovered equally well from a mild form of whole heart ischemia however hearts from diabetic animals recovered less well from a more severe ischemia. Tissue levels of the free fatty acid metabolites long chain acyl CoA and long chain acyl carnitine esters were elevated in hearts from diabetic animals. The failure of hearts from diabetic animals was associated with a greater increase in acyl CoA and acyl carnitine esters than occured in hearts from non-diabetic animals. These observations support the suggestion that the diabetic myocardium is able to function adequately under submaximal conditions and that its early failure when exposed to severe ischemia or high work loads may result from an altered metabolic state.

Ingebretson et al. (1980),using alloxan diabetic rats maintained on insulin for at least 2 weeks and then withdrawn 4 days prior to

experiments, observed, in isolated perfused working hearts a decrease in basal left ventricular pressure development and the maximum rate of left ventricular pressure development (+DP/dt) when compared to controls. There was no difference in coronary flow or cardiac output of hearts from diabetic animals as compared to controls. Both groups recovered equally well following a ten minute period of anoxia however when the afterload was increased by 71%, hearts from diabetic animals demonstrated a decreased ability to recover from anoxia. This difference could not be overcome by increasing the extracellular glucose.

The observation of high levels of plasma free fatty acids in the hearts of diabetic subjects has prompted investigators to compare the diabetic myocardium with the ischemic myocardium. Shug et al. (1975) observed an increase in the concentration of long chain acyl CoA esters and a decrease in adenine nucleotide translocase in canine hearts following ischemia produced by ligation of the anterior coronary artery. Lopaschuck et al. (1981) reported that long chain acyl carnitines were increased in microsomal sarcoplasmic reticulum from 4 month alloxan and STZ diabetic rats. Similar increases in free fatty acid metabolites were reported by Feuvray et al. (1979), Denton and Randle (1967) and Randle et al. (1966).

5. Function of the Sarcoplasmic Reticulum in Diabetes

Diabetic hearts observed in vivo and in vitro have a decreased ability to relax (Regan et al. 1981, Vadlamudi and McNeill 1981a). The sarcoplasmic reticulum is important in modulating cardiac relaxation (Tada et al. 1978). Penpargkul et al. (1981) reported a decreased

calcium uptake into sarcoplasmic reticulum from hearts of 4 to 9 week STZ diabetic rats. The activities of Mg^{++}-ATPase and (Ca^{++} + Mg^{++})-ATPase were also depressed. A depression of calcium transport was also observed by Lopaschuk et al. (1981) in cardiac sarcoplasmic reticulum from 4 month alloxan and STZ diabetic animals. Calcium transport was depressed in sarcoplasmic reticulum from control and diabetic hearts by μM concentrations of palmityl carnitine, the most abundant of the long chain acyl carnitines. The authors suggested that the decreased calcium uptake might result from an inhibitory influence of the long chain acyl carnitines which they reported to be elevated in the diabetic hearts. This hypothesis provides a link between the metabolic derangement of the diabetic heart and the observation of depressed relaxation.

6. The Effect of Diabetes Mellitus on the Contractile Units of the Myocardium

The functional changes observed in the diabetic myocardium might result from a modification of myocardial contractile elements. Malhotra et al. (1981) reported that the activities of Ca^{++} ATPase from cardiac actomyosin as well as Ca^{++} ATPase and actin activated ATPase from pure myosin are significantly depressed in rats as little as one week after the induction of STZ diabetes. Pierce and Dhalla (1981) observed a decrease in myofibrillar Ca^{++} ATPase activity in rats eight weeks after the induction of diabetes with STZ. Fein et al. (1981) reported that insulin therapy in 6 to 10 week STZ diabetic rats caused a gradual recovery of actomyosin and myosin ATPase activities, however Dillman (1980) reported a significant depression Ca^{++} ATPase from actomyosin

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and myosin of 8 week STZ diabetic rats which had been maintained on insulin for the final 4 weeks.

7. Summary

Diabetes mellitus produces a change in the metabolic state of many tissues. Free fatty acid oxidation is enhanced in the diabetic myocardium. Relaxation is impaired in hearts from chronically diabetic animals and this results in increased stiffness. This defect may be due to enhanced levels of connective tissue or to a defect in calcium uptake by SR. The diabetic heart also appears to have an altered sensitivity to both adrenergic and cholinergic agents possibly as a consequence of autonomic neuropathy. Increased workloads and periods of sustained anoxia are tolerated less well by hearts from diabetic animals than controls. There is some evidence that diabetes produces a defect in the contractile machinery of diabetic hearts.

II. Cardiac Glycosides and the Heart

1. Introduction

The observation that the high frequency of congestive heart failure among diabetics could not be explained by known risk factors led Kannel et al. (1974) to suggest that "...diabetes is another discrete cause of congestive heart failure and that some form of cardiomyopathy is associated with diabetes...". Despite the use of cardiac glycosides and potent diuretics congestive heart failure remains a dangerous and very often lethal condition.

The medicinal value of cardiac glycosides has been recognized since ancient times. The Egyptians, Chinese and Romans utilized these drugs for their therapeutic as well as toxic properties. These drugs are today used in the treatment of atrial fibrillation and flutter, paroxysmal tachycardia, sick sinus syndrome and, most importantly, in the treatment of congestive heart failure. This is a common end point for many cardiovascular disorders such as atherosclerosis or rheumatic myocarditis and may occur following a myocardial infarction (Moe and Farah, 1975).

The failing heart has a decreased capacity to develop force during systole. This results in a lowering of the Starling Curve. For a given cardiac output, the failing heart must develop a greater end diastolic pressure than a healthy heart. The maximum cardiac output of a failing heart is much less than that of a normal heart. The inefficient failing heart fails to empty totally with each contraction. For a given pressure the volume of a failing heart is much greater than that of a healthy heart. Despite the body's attempt to compensate for the decrease in ejection volume by increasing heart rates the

cardiac output remains reduced in congestive heart failure. There is an inadequate perfusion of organs (Moe and Farah, 1975).

Cardiac glycosides are of value in the treatment of heart failure due to their direct positive inotropic action which causes the ventricle to develop more tension and eject more fluid vs. a given after load. The increase in stroke volume and hence, cardiac output allows the heart to empty more adequately and leads to a decrease in end systolic and end diastolic volumes (Moe and Farah, 1975).

2. Role of $(Na^+ + K^+)$-ATPase in the Positive Inotropic Action of Cardiac Glycosides

The effect of cardiac glycosides observed _in vivo_ results from actions of these drugs on mechanical and electrical properties of the myocardium as well as actions on the nerves which innervate the heart. The use of isolated tissue preparations has enabled investigators to examine the effect of digitalis in the absence of innervation.

The sodium pump is responsible for actively transporting Na^+ and K^+ against their electrochemical gradients and thereby allowing cells to maintain cytoplasmic concentrations of Na^+ less than and K^+ greater than those in the extracellular fluid. In 1957 Skou described an ATPase from crab nerve membranes which was stimulated by Na^+ and K^+ in the presence of Mg^{++} and suggested that this enzyme could provide the physiological mechanism for maintaining the low cytoplasmic concentrations of Na^+ and high cytoplasmic concentrations of K^+ (Skou, 1957). In the quarter century since this observation, it has become quite firmly established that $(Na^+ + K^+)$-ATPase is indeed the sodium pump.

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Cardiac glycosides are potent and specific inhibitors of $(Na^+ + K^+)$-ATPase. In 1960 Skou reported that the Na^+ plus K^+ stimulated ATPase activity which he had previously described, could be inhibited in a dose dependent manner by ouabain (Skou, 1960). Ouabain, and to a lesser extent other cardiac glycosides, have been utilized as tools in the study of the enzyme. Robinson and Flashner (1979) stated, "ouabain inhibits the sodium pump, and those fluxes that ouabain inhibits are fluxes through the sodium pump".

The sodium pump is generally thought to expend energy for the movement of K^+ in and Na^+ out of cells. Glynn et al. (1975) have reported that, in erythrocytes, the pump can exist in four transport modes all of which are sensitive to ouabain. These are i) coupled Na^+/K^+ exchange, ii) uncoupled Na^+ efflux, iii) Na^+/Na^+ exchange and iv) K^+/K^+ exchange. In terms of maintaining desired Na^+ and K^+ gradients, the coupled Na^+/K^+ exchange mode is the most important. The reported stoichiometry varies with enzyme source, however, the erythrocyte pump appears to operate with $3Na^+/2K^+/ATP$. (Sen and Post, 1964). The resulting net outward movement of positive change contributes to the resting membrane potential of the cell.

There is little doubt that ouabain and other cardiac glycosides are capable of inhibiting $(Na^+ + K^+)$-ATPase. There remains, however, some question as to the relationship between this inhibition and the positive inotropic effect of cardiac glycosides on myocardial tissues. It has been reported that very low concentrations of cardiac glycosides may stimulate the sodium pump and still produce a positive inotropic effect (Godfraind and Ghysel-Burton, 1977 and Ghysel-Burton and Godfraind, 1979). This dilemma will be addressed

in a later section. The majority of evidence to date links the inotropic effect to an increase in exchangeable calcium which has been observed following exposure of cardiac tissues to glycosides. In 1964 Langer proposed the existance of a link between intracellular Na^+ concentration and Ca^{++} influx. Considerable evidence suggests that such a Na^+/Ca^{++} exchange mechanism does exist in cardiac tissue. It has been proposed that, in response to cardiac glycoside inhibition of $(Na^+ + K^+)$-ATPase, there is an increase in intracellular Na^+ leading to an increase in Na^+/Ca^{++} exchange and therefore an increase in intracellular Ca^{++} concentration (Langer and Serena, 1970). This Ca^{++} may be available to interact with the contractile elements and produce the inotropic event. This hypothesis has become very popular however a definitive proof of its validity has eluded investigators.

The inotropic response to cardiac glycosides has a very slow onset. A possible explanation could be that $(Na^+ + K^+)$-ATPase is a carrier of cardiac glycosides, moving the drugs from the extracellular fluid to intracellular sites. A recent paper by Yamamoto et al. (1981) provides strong evidence against this hypothesis. They reported that the affinity of $(Na^+ + K^+)$-ATPase for ouabain was almost the same when prepared from guinea pig left atria, right ventricle or papillary muscles. The number of glycoside binding sites per unit of protein and the $(Na^+ + K^+)$-ATPase activity were greater in preparations from right ventricle or papillary muscles than those from left atria homogenates. Ouabain-sensitive ^{86}Rb uptake into intact cells, an index of sodium pump activity, was greater in papillary muscle preparations than in left atrial preparations. The rate of onset of the positive inotropic response to ouabain was not different

in right ventricle and papillary muscles compared to left atria despite the higher $(Na^+ + K^+)$-ATPase concentration and greater capacity for active transport of monovalent cations in these two preparations as compared to left atria. If the enzyme was the transporter of the glycosides, one would predict that the onset of ouabain-induced inotropy would be more rapid in tissues displaying higher concentrations of enzyme. The magnitude of the response should be related to the concentration of enzyme. The inotropic response to ouabain was greater in left atria and right ventricle as compared to papillary muscles while the enzyme concentration was greater in both right ventricle and papillary muscles than in left atria.

If $(Na^+ + K^+)$-ATPase is the mediator of the positive inotropic effect of cardiac glycosides in cardiac tissues, one would expect to find the enzyme in such tissues and indeed there is little doubt regarding its presence. Furthermore, these drugs should bind specifically on or near the enzyme. In 1974 Ruoho and Kyle employed photo labelling techniques to demonstrate the digitalis binding site on the α subunit of $(Na^+ + K^+)$-ATPase. Using photo-affinity labelling and other techniques investigators are now attempting to define the molecular characteristics of the binding sites for cardiac glycosides on $(Na^+ + K^+)$-ATPase.

If enzyme inhibition is the cause of the inotropic response, enzyme inhibition should be detected before, or at least at the same time as, the inotropic response is observed. Examining the data, one is faced with technical problems of enzyme assays and data which have not been independently confirmed. Okita and co-workers

reported no change in enzyme activity following 3 hour incubation with a concentration of cardiac glycoside which produced a 50-80% increase in tension.(Roth-Schechter et al., 1970). In a later paper Okita's group was able to demonstrate enzyme inhibition which persisted following a washout of drug which was accompanied by a loss of the inotropic effect (Okita et al., 1973). The observations of Bentfeld et al. (1977) support these observations however enzyme inhibition was reversible at stimulation frequencies less than 4 Hz. At greater stimulation frequencies the inhibition became irreversible. It is possible that the continued inhibition of the enzyme could result from hypoxia due to cardiac glycoside induced increases in force of contraction and vasoconstriction. Akera et al. (1973) suggested that failure to detect enzyme inhibition at the time of the maximum inotropic action could be attributed to the observation that the half life of dissociation of the drug-enzyme complex is close to the half life of the offset of the inotropic response.

As stated earlier, there is little doubt that $(Na^+ + K^+)$-ATPase is the sodium pump. If enzyme inhibition is responsible for the inotropic response to cardiac glycosides, this response should be accompanied by Na pump inhibition. Ouabain sensitive ^{86}Rb uptake into cells preloaded with Na, is reduced when guinea pig ventricular slices are prepared during the inotropic response that follows cardiac glycoside administration (Akera et al., 1975). As the inotropic effect increases, so does the pump inhibition. Hougan and Smith (1978), using biopsy techniques, reported a decrease in sodium pump activity concomittant with an increase in maximum +DP/dt.

If cardiac glycosides produce their positive inotropic effect as a result of $(Na^+ + K^+)$-ATPase inhibition it would follow that any drug which inhibits the enzyme should also evoke a positive inotropic response. A number of agents have been shown to produce such an effect in cardiac tissues at concentrations that inhibit $(Na^+ + K^+)$-ATPase. These include N-ethylmaleimide, p-chloromercuri-benzoate, prednisolone, 3,20-bisguanylhydrazone, ethacrynic acid, fluoride, doxorubicin, sanguinarine, cassain, Rb^+ and Tl^+ (Akera and Brody, 1978).

Akera et al. (1975) demonstrated that, over a period of 60 minutes, the amount of digitoxin bound to the enzyme correlated very well with the change in contractile force. Digitoxin binding increased with contractile force over a 20 minute period of drug exposure and then declined with contractile force over a 40 minute washout period. This experiment was carried out on Langendorff guinea pig hearts.

Rhee et al. (1976) reported that $(Na^+ + K^+)$-ATPase activity could not be significantly reduced by concentrations of ouabain which produced an inotropic effect and only by concentrations which produced a toxic effect. The authors suggested that this supports a dissociation between the inotropic effect and enzyme inhibition. It should be noted that the enzyme activity was slightly reduced by the lower concentrations of ouabain. The authors used very small groups (n = 4 to 6) and actually observed a depression of $(Na^+ + K^+)$-ATPase activity, albeit not statistically significant, associated with the inotropic activity.

Recent work from Godfraind's lab has suggested that inhibition of the sodium pump may not be the sole determinant in the positive inotropic effect of ouabain in guinea pig hearts. Two specific binding sites for ouabain have been reported (Godfraind et al., 1980). Low doses of some glycosides, (those with an unsaturated lactone at the C17 position) stimulated the sodium pump and produced a positive inotropic response in guinea pig atria. When the concentration of K^+ in the buffer was changed there was a change in the ouabain ED50 for pump inhibition but not for the inotropic effect (Ghysel-Burton and Godfraind, 1979). Godfraind and Ghysel-Burton (1980) also plotted the positive inotropic effect vs. pump inhibition (as measured by ouabain sensitive $^{42}K^+$ uptake). They reported identical regression lines using various low K^+ solutions and in the presence of μmolar concentrations of dihydroouabain, a cardiac glycoside which does not contain an unsaturated lactone ring. The regression line for ouabain was much steeper suggesting that there may be an additional factor contributing to the inotropic response.

3. <u>Low Dose Effects of Ouabain - Possible Biphasic Inotropic Response in Cardiac Tissues</u>

There have, recently, been reports of biphasic responses to ouabain. Hougen and Smith (1980) reported that nmolar doses of ouabain could stimulate ouabain sensitive ^{86}Rb uptake in guinea pig left atria however this could be blocked by $10^{-6}M$ propranolol or pretreatment with reserpine. The authors suggested that the

stimulation of the sodium pump caused by low concentrations of cardiac glycosides could be mediated by the release of endogenous catecholamines. Recently Grupp et al. (1982) reported that atrial and ventricular tissues from guinea pigs, rabbits and cats displayed no inotropic response to low concentrations of ouabain. Schwartz et al. (1981) were unable to detect a biphasic inotropic effect in left atria or right ventricular papillary muscles from guinea pig, cat or rabbit. Ouabain evoked a monophasic inotropic response in left atria from rats however right ventricular strips from this species displayed a biphasic response to this drug. The low dose response represented 20-40% of the total inotropic response. The response was not altered by β blockade. The authors suggested that ouabain has two binding sites and that the binding to the high affinity site leads to a direct increase in intracellular calcium while binding to the low affinity site requires inhibition of $(Na^+ + K^+)$-ATPase and an increase in intracellular Na^+ which then produces an increase in intracellular Ca^{++}. In both mechanisms the increase in intracellular calcium leads to contraction. The authors also speculate that both of these mechanisms may be present in all species however the affinity of the two receptors for cardiac glycosides is so similar that they cannot be distinguished. Erdmann et al. (1981) reported a single ouabain binding site in human, cat, calf and dog cardiac tissues and two binding sites in guinea pig and rat hearts. They reported that ^{86}Rb uptake and $(Na^+ + K^+)$-ATPase

were inhibited only when ouabain was present in sufficient concentrations to occupy the low affinity sites. Erdmann et al. (1980) attempted to correlate [^{3}H] ouabain binding on isolated cardiac cell membranes and intact contracting tissue to ouabain induced inhibition of (Na$^+$ + K$^+$)-ATPase and ^{86}Rb uptake and to ouabain induced positive inotropy. These experiments were carried out on rat hearts. They reported two ouabain binding sites in cell membrane preparations and only one in intact ventricular tissue. The high affinity low capacity site in membranes has a KD (1.05 x 10^{-7}M) very similar to that of the single site in ventricular tissue (3 x 10^{-7}M). Half maximal inotropic effect occured at 3 x 10^{-7}M ouabain. These authors demonstrated a maximum inotropic effect of ouabain at 10^{-5}M while other authors (Ghysel-Burton and Godfraind, 1979 and Ku et al., 1976) reported a maximum at ouabain concentrations of 10^{-4}M. This discrepancy is very important in light of the very narrow dose range of the ouabain dose response curve. Erdmann et al. (1980) suggested that the low affinity, high capacity binding site (KD' = 2.8 x 10^{-5}M) observed in membrane tissues may be an artifact or may represent sites unrelated to the positive inotropic effect of cardiac glycosides.

Grupp, Grupp and Schwartz (1981) recently reported that a monophasic inotropic response was evoked by ouabain in rat left atria however a biphasic response to this drug could be observed

in rat ventricular strips. The low dose response had an ED50
of 0.5 μM and represented about 30% of the total ouabain response.
The response elicited by higher doses of ouabain had an ED50 of
35 μM and represented the remaining 70% of the response. The "overall"
ED50 was 16 μM ouabain. Reserpinization, α and β blockade and
histamine H_2 blockade had no effect on either response. The low
dose response was abolished when tissues were subjected to a ouabain
dose response curve, washed for 60 to 120 minutes and another dose
response curve was performed. The authors explained that this was
due to desensitization. The authors also noted that the ED50 for
the high dose effect was very close to the reported value for I50
of $(Na^+ + K^+)$ATPase and therefore the inotropy observed following
administration of high doses   might result from inhibition of
the enzyme. The low dose inotropic effect occurs when ouabain is
bound to the enzyme but the enzyme is not inhibited. The tension
development did not fall to predrug levels before the second dose
response curve was performed. It is possible that 60-120 minute washing
was not sufficient to remove ouabain from the high affinity sites.
If these sites were fully occupied, one would not expect to observe
an inotropic response to low concentrations of ouabain. Wellsmith
and Lindenmayer (1980) reported two conformations of $(Na^+ + K^+)$-ATPase
in canine sarcolemma. Ouabain was bound to both enzyme conformations,
however only one conformation was involved in the production of the
inotropic response.

The action of low doses of cardiac glycosides is poorly understood.
At present there is no clear line through the conflicting reports.

Further evidence is needed to define the inotropic response and to determine the link with sodium pump inhibition.

4. Low Sensitivity of Rat Hearts to Cardiac Glycosides

The ubiquitous nature of $(Na^+ + K^+)$-ATPase has allowed investigators to examine the action of cardiac glycosides in a number of species. The myocardium of rats is notoriously insensitive, requiring very high concentrations of glycosides to elicit a response (Repke et al., 1965). Investigators have attempted to explain the relative insensitivity of the rat myocardium.

An increase in stimulation frequency causes an increase in the force of myocardial contraction in several species including guinea pig and rabbit (Kruta, 1937 and Katzung et al., 1957). A similar change results in a decrease in the force of contraction in rat hearts (Benforando, 1958). Blesa et al., (1970) and Langer (1970) proposed that the mechanism responsible for this difference might also be responsible for the low sensitivity of the rat heart to cardiac glycosides. McCans et al. (1974) changed the positive staircase effect in the rabbit heart to a negative staircase effect by addition of the calcium channel blocker, verapamil. This treatment did not decrease the sensitivity of the rabbit heart to ouabain indicating that the absence of the Bowditch phenomenon was not sufficient for loss of sensitivity to ouabain.

In 1969 Allen and Schwartz reported differences in the binding characteristics of ouabain to $(Na^+ + K^+)$-ATPase preparation from rat, dog and beef sources. ^3H glycoside bound to dog and beef preparations in a 1:1 ratio while rat demonstrated a 2:1 to 3:1 ratio.

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The ouabain-enzyme complex from dog and beef was time and temperature sensitive while that from rat was not. Enzyme-drug complexes from rat tissues were disrupted by resuspension while those from dog and beef were not, indicating that the drug bound more loosely to $(Na^+ + K^+)$-ATPase isolated from rat tissues. The authors suggested that, in the rat, "the complex formed between the drug and one possible receptor is unstable and the drug probably binds to some sites unrelated to enzyme inhibition".

Tobin and Brody (1972) confirmed that the enzyme-ouabain complex was much less stable with rat $(Na^+ + K^+)$-ATPase than with other species. Tobin et al. (1972) suggested that species differences in sensitivity to cardiac glycosides were due to differences in the dissociation constants for the drug-enzyme complex. They examined enzymes from guinea pig, dog and cat but not rat as they were unable to demonstrate reproducible specific binding. If there were no difference in association rate constants and an increase in dissociation constant of enzyme-ouabain complexes in rats with respect to more sensitive species, one would expect that a steady state level of complex would be reached at an earlier time in rats. A higher concentration of ouabain would be required to reach the same steady state level as in more sensitive species. Ku et al. (1976) demonstrated that the positive inotropic effect of various concentrations of ouabain reached a plateau in less than 10 minutes in rat atria while tension continued to increase 30 minutes after exposure of guinea pig atria. This observation strengthens the argument that the differences in sensitivity to cardiac glycosides result from differences in dissociation rates.

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In 1979 Akera et al. compared ouabain with compounds with altered lactone or steroid configurations in terms of positive inotropic effect, time-response relationship and enzyme inhibition in guinea pigs and rats. The authors concluded that the low sensitivity of rat hearts to cardiac glycosides results from the absence of a lipid barrier which, in more sensitive species, stabilizes the drug-receptor complex. The absence of this barrier results in the increased dissociation constant of cardiac glycoside receptor in cardiac tissues of the rat.

5. Insulin and Diabetes and the $(Na^+ + K^+)$-ATPase Mediated Ouabain Response

Our initial interest in the response of the diabetic heart to ouabain stemmed from the observation of the increased risk of congestive heart failure in diabetics (Kannel et al., 1974). Cardiac glycosides are very valuable in the treatment of this disease and it was felt that their response might be altered. Several other observations suggested to us that diabetes might alter the response to these drugs.

Diabetes produces alterations in basement membranes of many tissues (Friedenwald, 1950, Bergstrand and Bucht, 1957, and Siperstein et al., 1968). Alterations in the environment near the $(Na^+ + K^+)$-ATPase could alter the interaction between enzyme and cardiac glycoside.

Baily and Dresel (1971) reported that the inotropic response of left atria from 3 day alloxan diabetic rabbits appeared initially similar to that of control atria, however, the response was not maintained unless insulin was added to the bathing medium. The authors

concluded that sugar transport and metabolism were necessary for maintenance of the positive inotropic response to cardiac glycosides. Resh et al. (1980) reported that insulin stimulated $(Na^+ + K^+)$-ATPase dependent $^{86}Rb^+$ uptake in rat adipocytes. These observations suggest that the positive inotropic effect of cardiac glycosides may be dependent on high basal $(Na^+ + K^+)$-ATPase activity such as that produced by insulin stimulation.

Onji and Liu (1980) reported that there was no difference in the number of ouabain binding sites in myocytes obtained from control and 5 to 8 day alloxan diabetic dogs. The authors did, however, observe a decrease in the affinity for K^+ in enzymes from diabetic animals. The effect would be amplified by the accompanied decrease in cellular K^+ content of diabetic tissues.

Ku (1980) reported a decrease in sodium pump activity in 5 week STZ diabetic rats. The same laboratory observed a decrease in the maximum inotropic response to ouabain of isolated left atria from these diabetic rats.(Sellers and Ku, 1981).

As mentioned previously, long chain acyl carnitine levels may be increased in the myocardium of diabetic animals (Shug et al., 1975). Adams et al. (1979) reported that increasing concentrations of palmityl carnitine will enhance and then inhibit the binding of ouabain to $(Na^+ + K^+)$-ATPase while all concentrations of palmityl carnitine will inhibit $(Na^+ + K^+)$-ATPase activity.

It appears that diabetes could interfere with the inotropic response to ouabain. It is possible that this could appear in early diabetes or it could appear as a result of progressive changes in the diabetic heart some of which has already been discussed.

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III. Summary of Experimental Aims

1. In light of the growing evidence for a specific cardiomyopathy associated with diabetes, it was of interest to us to examine the response of cardiac tissues from diabetic animals to the cardiac glycoside ouabain at various times after the induction of diabetes. We were interested in the qualitative nature of any change as well as in defining the time point at which such a change first appeared.

2. The observation of a biphasic response to ouabain in papillary muscles led us to investigate more closely, the effect of low concentrations of cardiac glycosides on the inotropic state of cardiac tissues.

3. Autonomic dysfunction is common among diabetics. Using isoproterenol, we hoped to define a time point at which this dysfunction became evident. We wished to compare this time point with that of the appearance of changes in the response to cardiac glycosides.

MATERIALS AND METHODS

1. Induction of Diabetes

Female Wistar rats of 175-250 g were made diabetic by a single dose of alloxan, 40-65 $mgkg^{-1}$, or streptozotocin (STZ), 50-60 $mgkg^{-1}$. The drugs were prepared in 0.1 M citrate buffer pH 4.5. Control animals received an injection of buffer. Animals were anaesthetized by exposure to ether fumes inside a bell jar. The tail of each animal was dipped in hot water ($\sim$60°) for approximately 30 seconds then wiped briskly with a Kim Wipe soaked with ethanol. This procedure dilated the lateral tail veins facilitating the intravenous administration of diabetogen or vehicle. The solutions were administered as rapidly as possible using a 1 ml syringe with a 25 gauge needle.

Animals were tested for glycosuria using Lilly Tes-Tape[R]. A reading of 4+ corresponding to a urine glucose concentration of 2% or more, was considered evidence of diabetes.

2. Maintenance of Animals

Animals were housed in wire cages containing 6 to 8 animals. They were provided with food (Purina rat chow) and water ad libitum. Approximately 18 hours prior to sacrifice food was withdrawn.

Animals were stunned by a blow to the head and killed by cervical dislocation. Blood was collected and the serum was separated by centrifugation and stored at -40 for later analysis of glucose and insulin. Hearts were quickly removed and placed in Chenoweth-Koelle (CK) buffer

pH 7.4 gased with 95% O_2/5% CO_2 at room-temperature. The buffer contained in mM: NaCl 120; KCl 5.6; $CaCl_2$ 2.2; $MgCl_2$ 2.1; glucose 10.0; $NaHCO_3$ 19.2 (Chenoweth and Koelle, 1946).

3. Preparation of Isolated Tissues

Left and right atria were separated from ventricular tissue. Papillary muscles were excised from the left ventricle. Branched papillaries were discarded. Left and right atria were separated and trimmed of extraneous tissue and care was taken to not damage the sino atrial node. All tissues were suspended in 25 ml tissue baths containing CK buffer pH 7.4 at 37° and bubbled with 95% O_2/5% CO_2.

Tissues were connected at one end via one or more pins to platinum stimulating electrodes and at the other end via a Palmer clip and silk suture to a Grass force displacement transducer. The transducers were coupled to a Beckman R611 Dynograph or to a Grass 79D Polygraph.

All tissues were adjusted to a diastolic tension of 1.0 g. Right atria were allowed to beat spontaneously. Left atria were equilibrated for 15 minutes and then stimulated with 5 msec square wave pulses at 3.3 Hz using a Grass stimulator S6C or SD9. Papillary muscles were immediately stimulated with similar pulses at 1 Hz. All tissues were equilibrated for approximately 60 minutes unless otherwise stated. Tissues were washed with warm fresh oxygenated buffer every 20 minutes except during a ouabain dose-response curve which took 2 hours to complete. The tissues could not be washed during this period. In the experiments where timolol was used for β blockade, 10^{-7} M timolol was present in the buffer at all times.

All dose response curves were performed in a cumulative manner using a buffer volume of 20 ml. Inotropic and chronotropic responses to isoproterenol reached a maximum in less than one minute. At the time of the maximum response the subsequent dose was added. The inotropic response to ouabain developed over a period of five to eight minutes. A period of ten minutes was allowed between the administration of each drug dose.

4. Preparation of Drugs

A 10^{-2} M stock solution of d,l isoproterenol was prepared in 0.05 M HCl at the beginning of each set of experiments. Dilutions were made in buffer immediately before each dose response curve. All ouabain solutions were prepared in buffer on the day of the experiment.

5. Analysis of Serum

Serum insulin concentrations were determined using a radioimmuno assay (RIA) kit purchased from Becton Dickinson. Serum glucose concentrations were determined using an Ames Blood Analyzer kit or a Glucose No. 510 (colorimetric) kit purchased from Sigma.

6. Statistical Analyses

The Students "t" test was used to compare a single experimental group to control. Analysis of variance was used when comparing three or more groups and significance was determined using the Newman-Keuls test. A value of $p < .05$ was considered to be significant in all experiments.

Materials

Alloxan monohydrate, streptozotocin, ouabain octahydrate and d,l isoproterenol HCl were purchased from Sigma. Timolol maleate was generously provided by Merck-Frosst.

RESULTS

I. Detection of Diabetes

Fasted serum glucose and insulin levels indicated that animals were diabetic by 7 days and remained diabetic past 70 days. At 7 days the serum glucose level of control animals was 70.1 ± 7.34 mg % while that of STZ diabetics was 305.4 ± 23.58 mg %. Serum insulin levels were 42.1 ± 5.08 μU ml^{-1} in samples from control animals and 18.50 ± 2.42 μU ml^{-1} in samples from STZ diabetic animals. At 70 days serum glucose levels from STZ diabetic rats were 477% of control levels.

II. Response to d,l Isoproterenol in Cardiac Tissues taken from Rats 7 or 70 Days after the Induction of Diabetes.

A. Inotropic Responses

Inotropic responses to isoproterenol were measured in left papillary muscles and left atria at both time points. In the absence of drug, there was no significant difference in the tension developed by control or diabetic tissues (Table I). Isoproterenol produced a dose-dependent increase in tension in both atria and papillary muscles. There was no significant difference in the response of papillary muscles from control or diabetic animals at 7 or 70 days (Fig 2 and 3). At 70 days the response of tissues from diabetic animals was consistently but not significantly smaller at each dose of isoproterenol (Fig 3). There was also no significant difference in the response of left atria from control or diabetic animals

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Table I

Inotropic Responses of Cardiac Tissues to d,l Isoproterenol

7 or 70 Days After the Induction of Diabetes.

Tissue	Experimental Group	n	Time Point (days)	Basal Developed Tension (g)	Maximum Developed Tension (g)	Maximum Increase in Tension (g)
papillary	control	8	7	0.49±0.11	0.87±0.17	0.38±0.08
muscles	alloxan	6	7	0.91±0.19	1.38±0.26	0.47±0.10
papillary	control	8	70	0.79±0.19	1.13±0.26	0.33±0.08
muscles	STZ	13	70	0.65±0.11	0.79±0.14	0.18±0.05
left	control	4	7	0.64±0.22	1.43±0.52	0.79±0.31
atria	STZ	4	7	0.75±0.08	1.36±0.09	0.61±0.08
	alloxan	5	7	0.60±0.05	1.30±0.14	0.66±0.12
left	control	5	70	0.72±0.20	1.31±0.20	0.59±0.06
atria	STZ	8	70	0.77±0.09	1.31±0.10	0.54±0.05

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Fig 2.

Inotropic Response of Papillary Muscles from Control and

7 Day Alloxan Diabetic Rats to d,l Isoproterenol.

Tissues were stimulated with square wave pulses at a

frequency of 1 hz and were equilibrated at 37° in

oxygenated CK buffer, pH 7.4, for 1 hour prior to drug

addition. Dose response curves were performed in a

cumulative manner.

Response is expressed in terms of increase in tension

(g) vs. the log of the molar concentration of

isoproterenol. Each point represents the mean ± SEM.

$^\Delta$Control n=8; ^{O}Alloxan n=6.

RESPONSE TO ISOPROTERENOL
(◄) CONTROL (N=8)
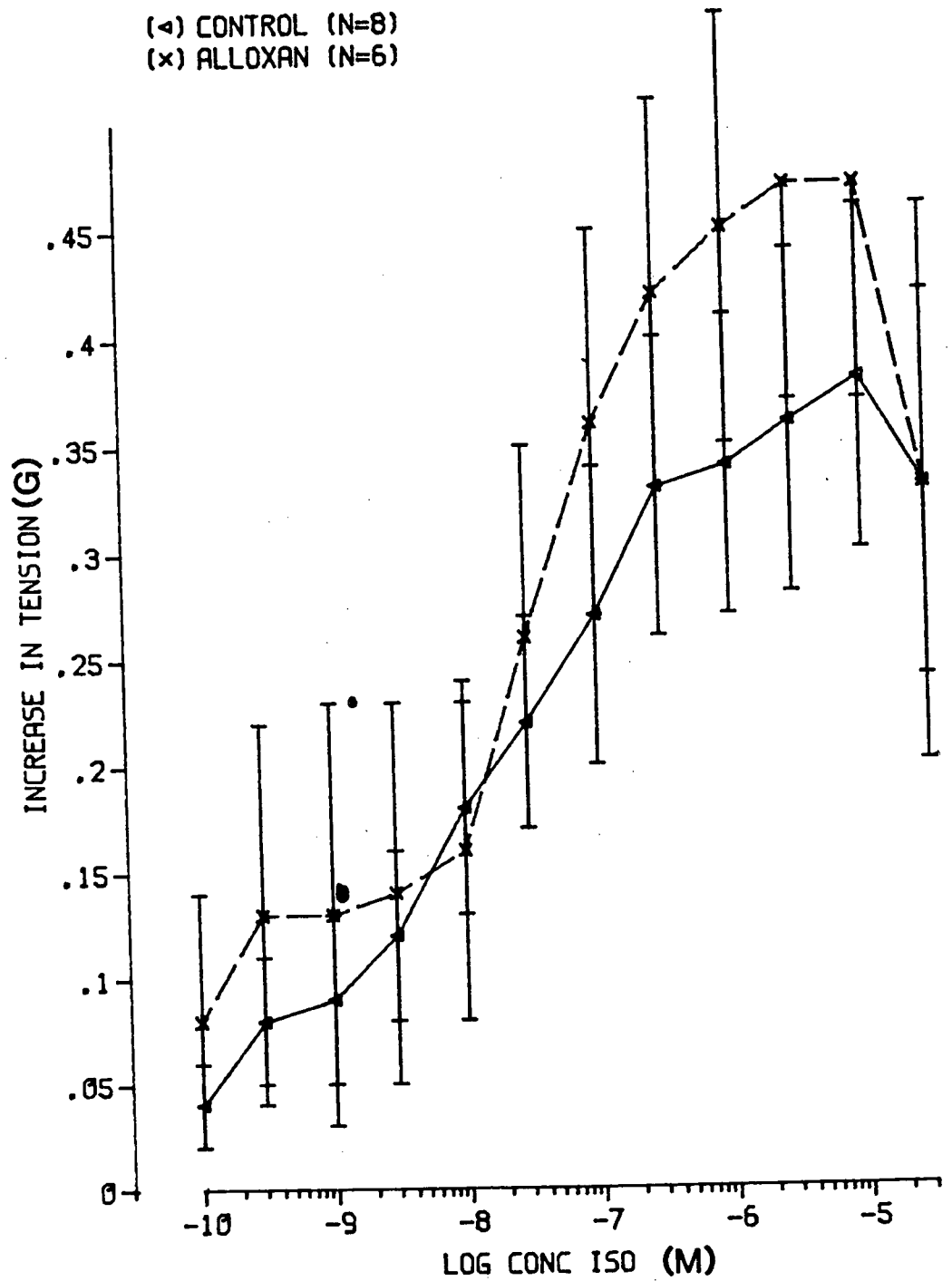(×) ALLOXAN (N=6)

FIG 2

RESPONSE TO ISOPROTERENOL
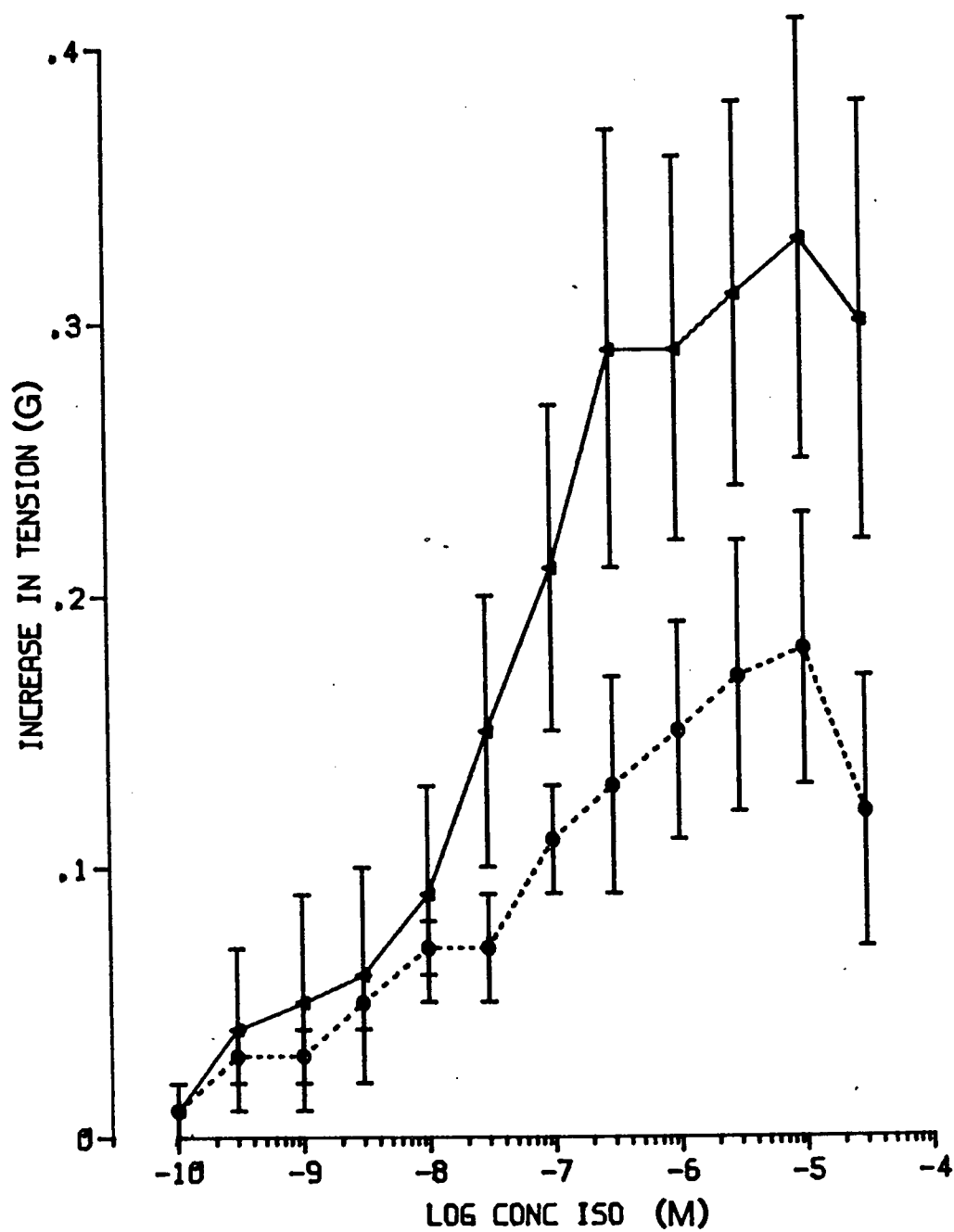(◄) CONTROL (N=8)
(o) STZ (N=13)

FIG 3

<u>Fig 3.</u>

Inotropic Response of Left Papillary Muscles from Control
and 70 Day Diabetic Rats to d,l Isoproterenol.

Tissues were stimulated with square wave pulses at a
frequency of 1 hz and were equilibrated at 37° in oxygenated
CK buffer, pH 7.4, for 1 hour prior to drug addition. Dose
response curves were performed in a cumulative manner.

Response is expressed in terms of increase in tension (g)
vs. the log of the molar concentration of isoproterenol.
Each point represents the mean ± SEM. ΔControl n=8;
^{O}STZ n=13.

to isoproterenol.at 7 or 70 days (Fig 4 and 5). At 70 days, there was
a consistent but not significant, reduction in the response of diabetic
tissues.

B. Chronotropic Responses

Chronotropic responses to isoproterenol were measured in right atria
from control and diabetic animals 7 and 70 days after the induction of
diabetes. Seven days after the induction of diabetes the basal rate was
significantly reduced in atria from STZ animals with respect to controls
(Table II). Atria from alloxan diabetic animals demonstrated a reduction
in basal rate which was not statistically significant. At 70 days there
was very little difference in the basal rate of atria from control and
diabetic animals. Despite the difference in basal rates, there was no
significant difference in the maximum rate attained or the maximum increase
in rate in atria from control and 7 day diabetic rats (Fig 6 a/b, Table II).
Seventy days after the induction of diabetes atria from diabetic rats
demonstrated smaller responses to isoproterenol than did atria from
control animals. Although this decrease was consistent throughout the
range of the dose response curve, the difference was not statistically
significant (Fig 7).

Fig 4.

Inotropic Response of Left Atrial Tissues from Control and
7 Day Diabetic Rats to d,l Isoproterenol.

Tissues were stimulated with square wave pulses at a
frequency of 3.3 hz and equilibrated, at 37°, in
oxygenated CK buffer, pH 7.4, for 1 hour prior to drug
addition. Dose response curves were performed in a
cumulative manner.

Response is expressed in terms of increase in tension (g)
vs. the log of the molar concentration of isoproterenol.
Each point represents the mean ± SEM. $^{\Delta}$Control n=4;
^{X}Alloxan n=5; ^{O}STZ n=4.

RESPONSE TO ISOPROTERENOL
(◄) CONTROL (N=4)
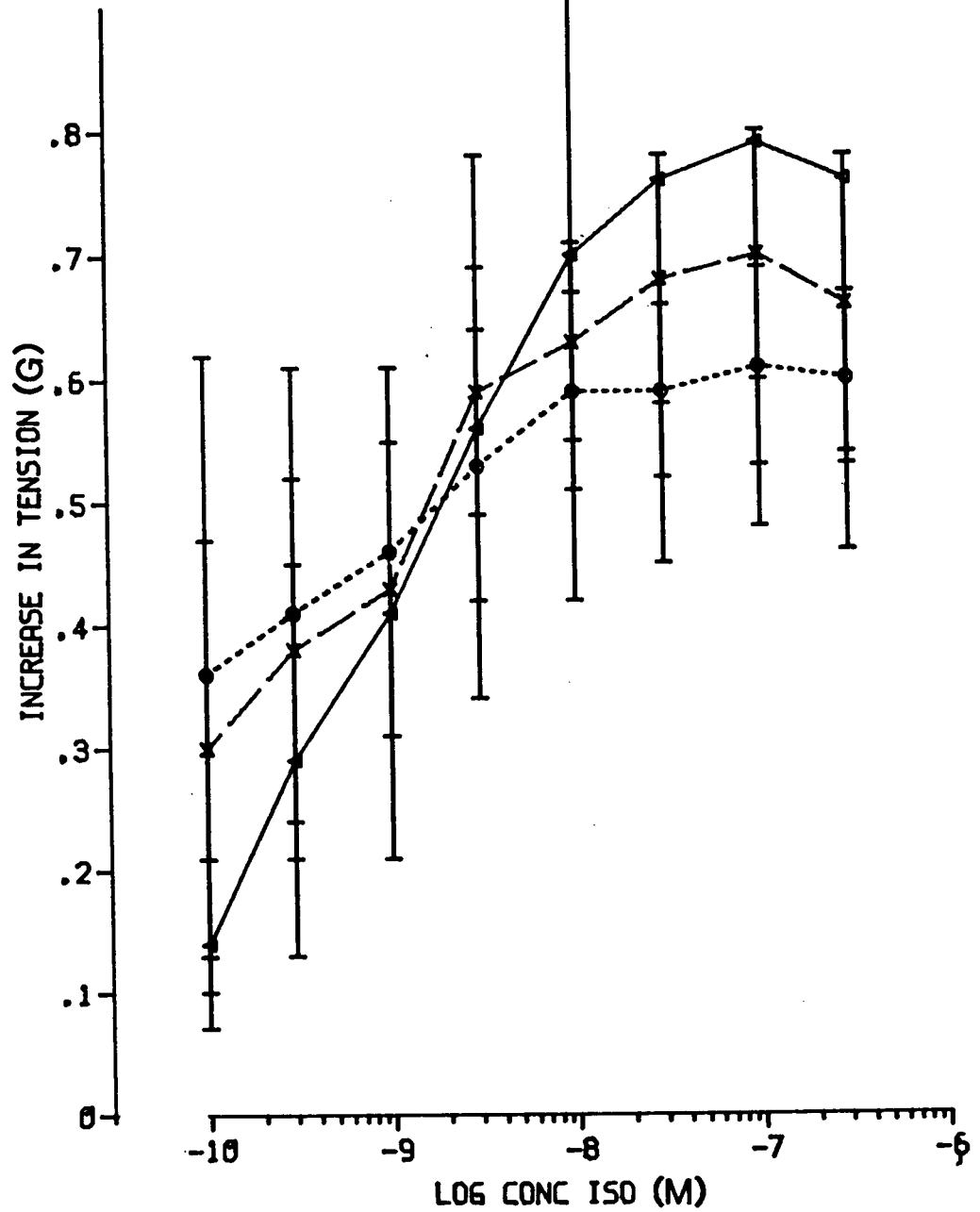(×) ALLOXAN (N=5)
(o) STZ (N=4)

FIG 4

Fig 5

Inotropic Response of Left Atria from Control and 70 Day
Diabetic Rats to d,1 Isoproterenol.

Tissues were stimulated with square wave pulses at a
frequency of 3.3 hz and equilibrated, at 37°, in oxygenated
CK buffer, pH 7.4, for 1 hour prior to drug addition. Dose
response curves were performed in a cumulative manner.

Response is expressed in terms of increase in tension (g)
vs. the log of the molar concentration of isoproterenol.
Each point represents the mean ± SEM. $^\Delta$Control n=5;
^{O}STZ n=8.

RESPONSE TO ISOPROTERENOL
(◄) CONTROL (N=5)
(o) STZ (N=8)
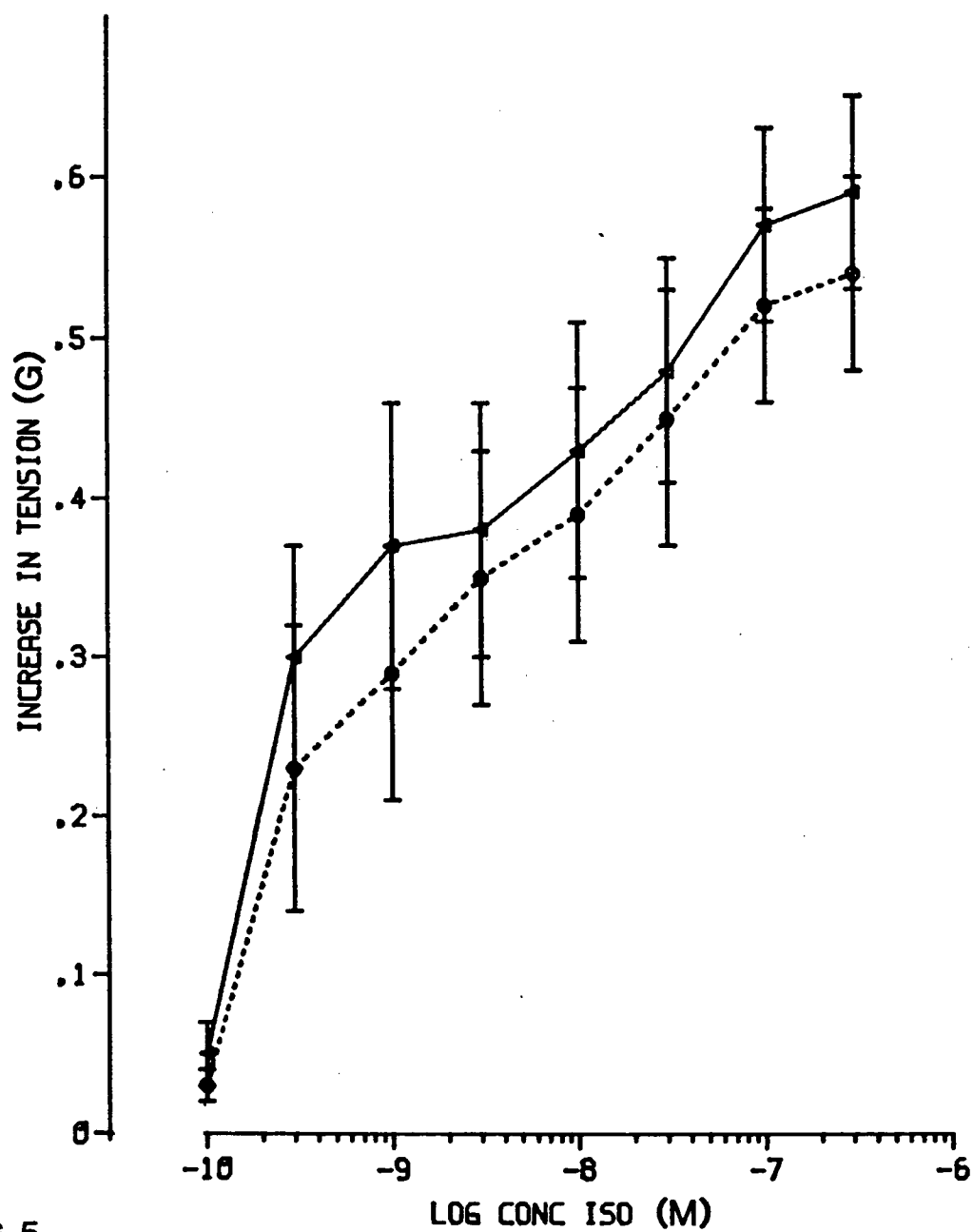
FIG 5

Table II

Chronotropic Responses of Right Atria to d,l Isoproterenol
7 or 70 Days After the Induction of Diabetes.

Experimental Group	n	Time Point (days)	Basal Rate (beats min^{-1})	Maximum Rate (beats min^{-1})	Maximum Increase in Rate (beats min^{-1})
control	4	7	281±20	418±11	137±25
STZ	3	7	208±7[a]	393±11	185±11
alloxan	5	7	225±17	389±19	164±9
control	5	70	223±10	439±16	217±11
STZ	7	70	235±18	403±10	159±25

[a] $p < .05$ compared to control

<u>Fig 6a and 6b</u>

Chronotropic Response of Right Atria from Control and
7 Day Diabetic Rats to d,l Isoproterenol.

Tissues were allowed to beat spontaneously and were
equilibrated, at 37°, in oxygenated CK buffer pH 7.4
for 1 hour prior to drug addition. Dose response curves
were performed in a cumulative manner.

The chronotropic response is expressed in beats min^{-1}.
In part "a" the data are expressed in terms of absolute
rate vs. the log of the molar concentration of isoproterenol.
In part "b" the data are expressed in terms of increase
in rate vs. the log of the molar concentration of
isoproterenol. Each point represents the mean ± SEM.
ΔControl n=4; ^{X}Alloxan n=5; ^{O}STZ n=3.

RESPONSE TO ISOPROTERENOL
(◄) CONTROL (N=4)
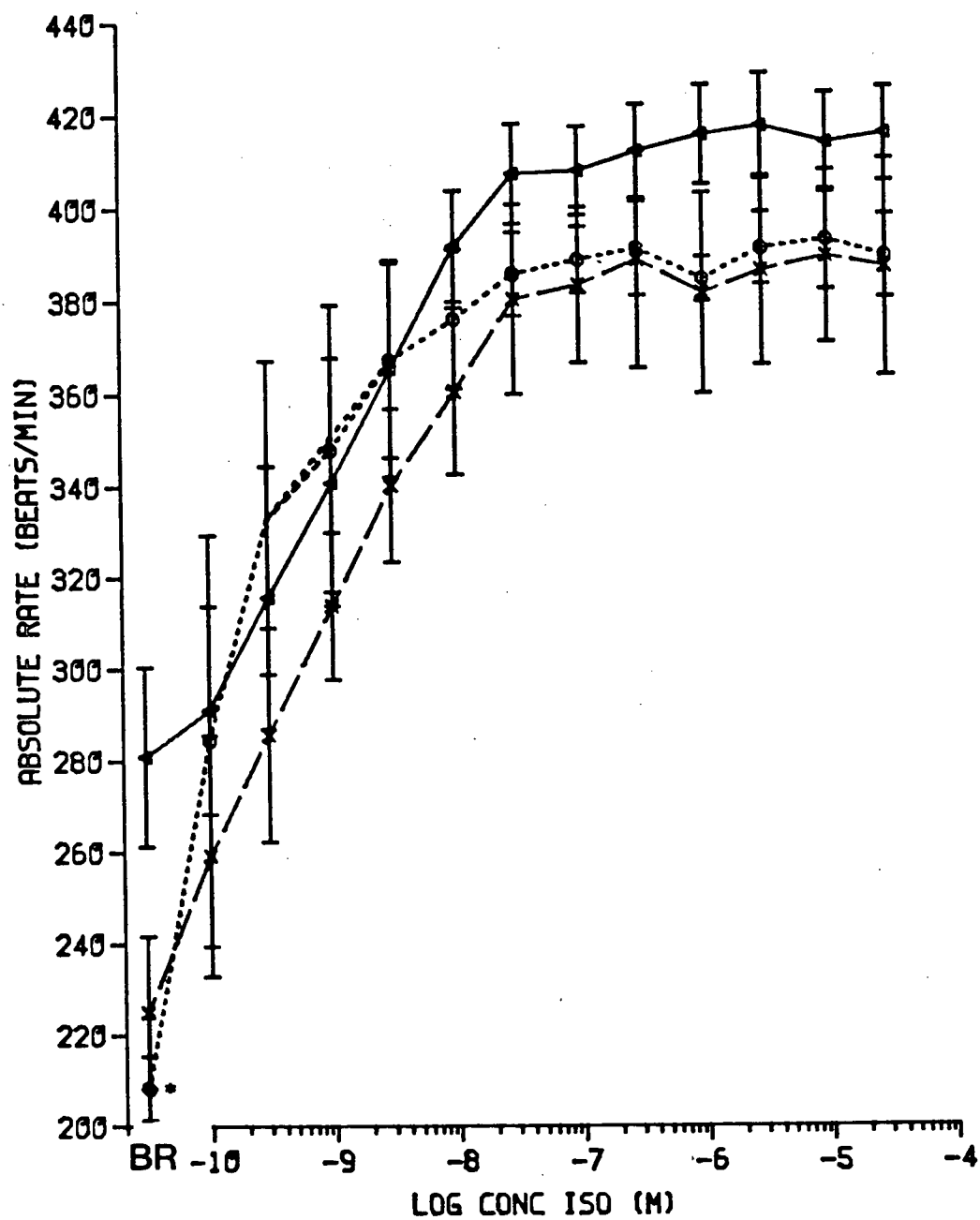(×) ALLOXAN (N=5)
(o) STZ (N=3)

FIG 6a

RESPONSE TO ISOPROTERENOL
(◄) CONTROL (N=4)
(×) ALLOXAN (N=5)
(o) STZ (N=3)
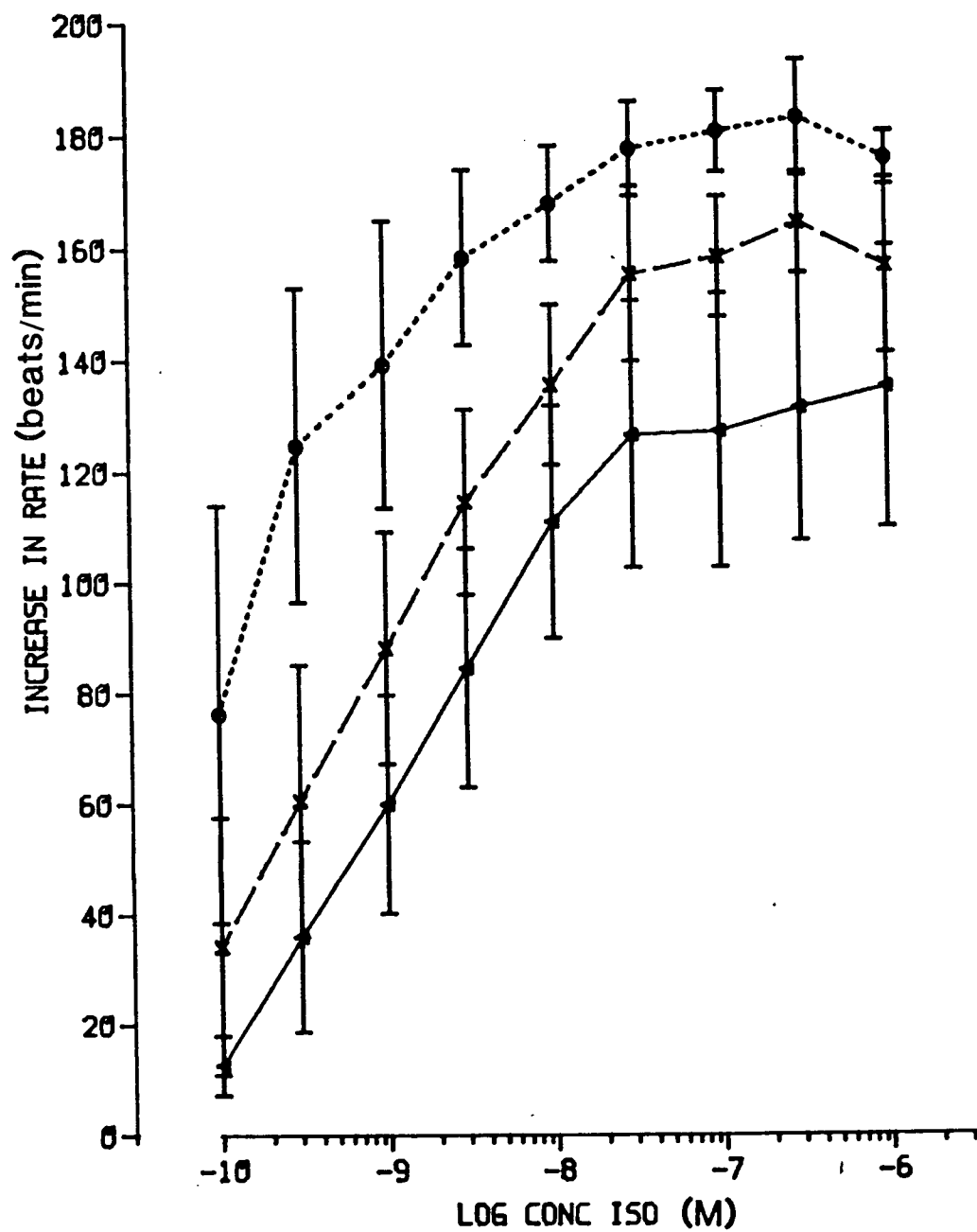
FIG 6b

Fig 7

Chronotropic Response of Right Atria from Control and
70 Day STZ Diabetic Rats to d,l Isoproterenol.

Tissues were allowed to beat spontaneously and were
equilibrated, at 37°, in oxygenated CK buffer pH 7.4
for 1 hour prior to drug addition. Dose response curves
were performed in a cumulative manner.

Positive chronotropic response is expressed in terms of
increase in rate beats min^{-1}) vs. the log of the molar
concentration of isoproterenol. Each point represents
the mean ± SEM. $^{\Delta}$Control n=5; ^{O}STZ n=7.

RESPONSE TO ISOPROTERENOL
(◄) CONTROL (N=5)
(o) STZ (N=7)

FIG 7

III Response to Ouabain in Cardiac Tissues 7 Days, 70 Days or 6 Months After the Induction of Diabetes
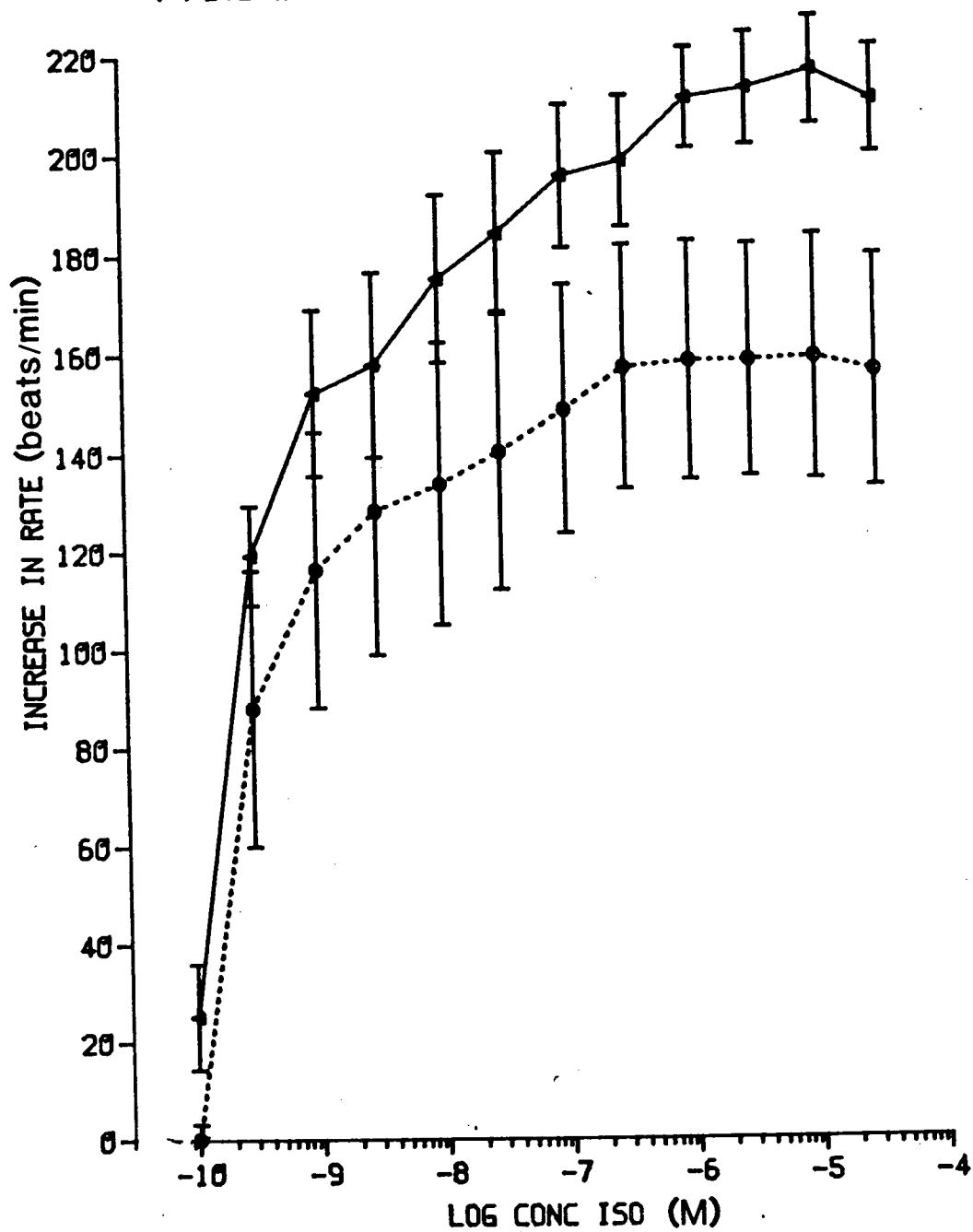
A. Effect of Timolol on Ouabain Dose Response Curves

The rat myocardium is notoriously insensitive to the effects of cardiac glycosides. Very high concentrations of these drugs are required to produce a positive inotropic effect. In order to determine whether release of endogenous catecholamines contributed to the observed inotropic effect, dose response curves to ouabain were compared in the presence and absence of the β blocker timolol. Timolol was present in the buffer, during the equilibration period and throughout the dose response curve, at a concentration (10^{-7} M) which had previously been shown to block the inotropic effect of catecholamines. In the presence of timolol the resting tension of left atria was slightly, but not significantly, greater than in the absence of this drug. As shown in Figure 8, there was no significant difference in ouabain dose response curves performed in the presence or absence of the β blocker.

B. Inotropic Responses to Ouabain in Left Atria and Papillary Muscles.

In the absence of ouabain, there was no statistically significant difference in the basal developed tension of tissues from control or diabetic animals (Table III). At the later time points (70 days and 6 months) there appeared to be a tendency for control tissues to develop slightly more tension. Seven days after the induction of diabetes ouabain produced a smaller increase in tension in left atria from both groups

Fig 8

Effect of β Blockade on the Positive Inotropic Response
to Ouabain in Left Atria from Rats.

Tissues were stimulated with square wave pulses at a
frequency of 3.3 hz. Prior to ouabain exposure, tissues
were equilibrated for 1 hour at 37° in oxygenated CK buffer,
pH 7.4, containing, where indicated 10^{-7}M timolol. Dose
response curves were performed in a cumulative manner.

Response of atria is expressed in terms of increase in
tension (g) vs. the log of the molar concentration of
ouabain. Each point represents the mean ± SEM. $^{\Delta}$CK n=7;
^{X}CK + 10^{-7} M Timolol n=8.

RESPONSE TO OUABAIN
(◄) CK (N=7)
(×) CK + TIM (N=8)

INCREASE IN TENSION (G)
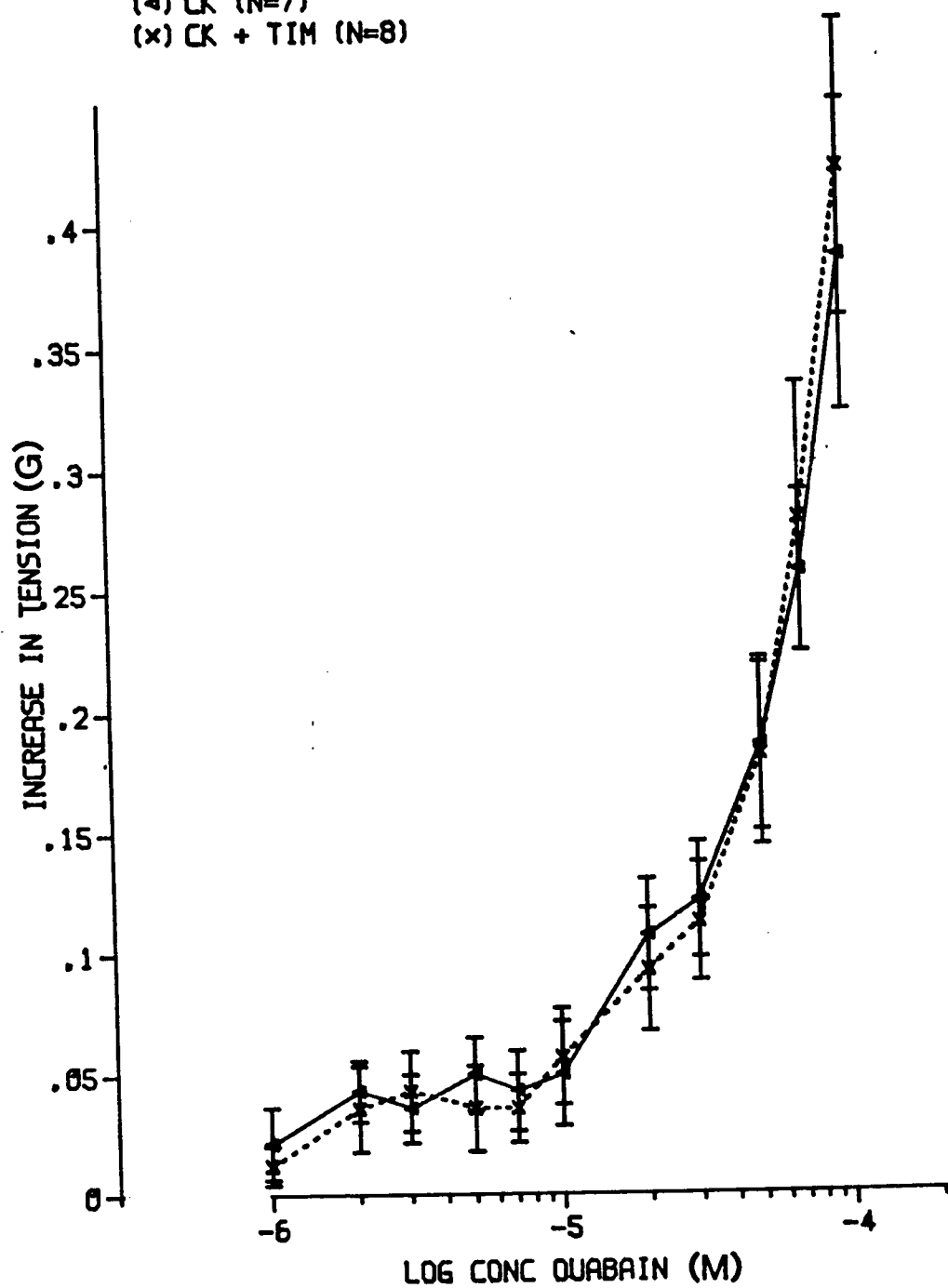
LOG CONC OUABAIN (M)

FIG 8

Table III

Inotropic Responses of Cardiac Tissues to Ouabain at Various Times
After the Induction of Diabetes.

Tissue	Experimental Group	n	Time Point	Basal Developed Tension (g)	Maximum Developed Tension (g)	Maximum Increase Tension (g)
left	control	6	7 days	0.42±0.09	0.75±0.16	0.33±0.09
atria	STZ	6	7 days	0.78±0.11	0.98±0.11	0.18±0.02
	alloxan	4	7 days	0.64±0.06	0.86±0.11	0.23±0.06
left	control	9	6 months	1.06±0.10	1.59±0.21	0.51±0.08
atria	STZ	4	6 months	0.70±0.12	0.90±0.11	0.21±0.03[a]
	alloxan	6	6 months	0.73±0.15	0.95±0.17	0.22±0.03[a]
papillary	control	15	7 days	0.30±0.05	0.77±0.09	0.47±0.06
muscles	STZ	8	7 days	0.34±0.03	0.66±0.07	0.32±0.06
papillary	control	5	70 days	0.76±0.20	1.29±0.29	0.56±0.08
muscles	STZ	12	70 days	0.53±0.10	0.77±0.14	0.26±0.07[a]

a = p<.05 compared to control.

of diabetic animals compared to control (Fig 9). This difference was evident at all points of the dose response curve except the two lowest doses, however at no point was the difference statistically significant. Six months after the induction of diabetes this pattern was again evident (Fig 10). Tissues from both diabetic groups demonstrated a smaller increase in tension with increasing doses of ouabain than did tissues from control animals. This depression of response was statistically significant in tissues from alloxan diabetic animals at doses of ouabain greater than 3×10^{-5} M. Similar results were obtained in left papillary muscles. Seven days after the induction of diabetes papillary muscles from diabetic animals consistently demonstrated a non-significant decrease in response to ouabain relative to controls (Fig 11). Seventy days after diabetes was induced, the response of papillary muscles from STZ diabetic animals was significantly less than that of control animals at doses of ouabain greater than 10^{-5} M (Fig 12).

IV. Effect of Time and β Blockade on the Positive Inotropic Effect of Ouabain on Cardiac Tissues.

In an attempt to explain the biphasic dose response curves to ouabain demonstrated by papillary muscles the following experiments were carried out. Left atria and papillary muscles were equilibrated for a period of one or three hours in the presence or absence of 10^{-7} M timolol. Prior to ouabain administration there was no significant difference in tension development among groups of atrial or papillary tissues. Dose response

<u>Fig 9</u>

Inotropic Response of Left Atria from Control and 7 Day
Diabetic Rats to Ouabain.

Tissues were stimulated with square wave pulses at a
frequency of 3.3 hz and were equilibrated, at 37°, for 1
hour in oxygenated CK buffer, pH 7.4. Isoproterenol
dose-response curves were performed, tissues were washed
for a period of 1 hour and ouabain dose response curves
were then obtained. Dose response curves were performed in
a cumulative manner.

Response is expressed in terms of increase in tension (g)
vs. the log of the molar concentration of ouabain. Each
point represents the mean ± SEM. $^\Delta$Control n=6; $^\circ$STZ n=6;
^{X}Alloxan n=4.

RESPONSE TO OUABAIN
(◄) CONTROL (N=6)
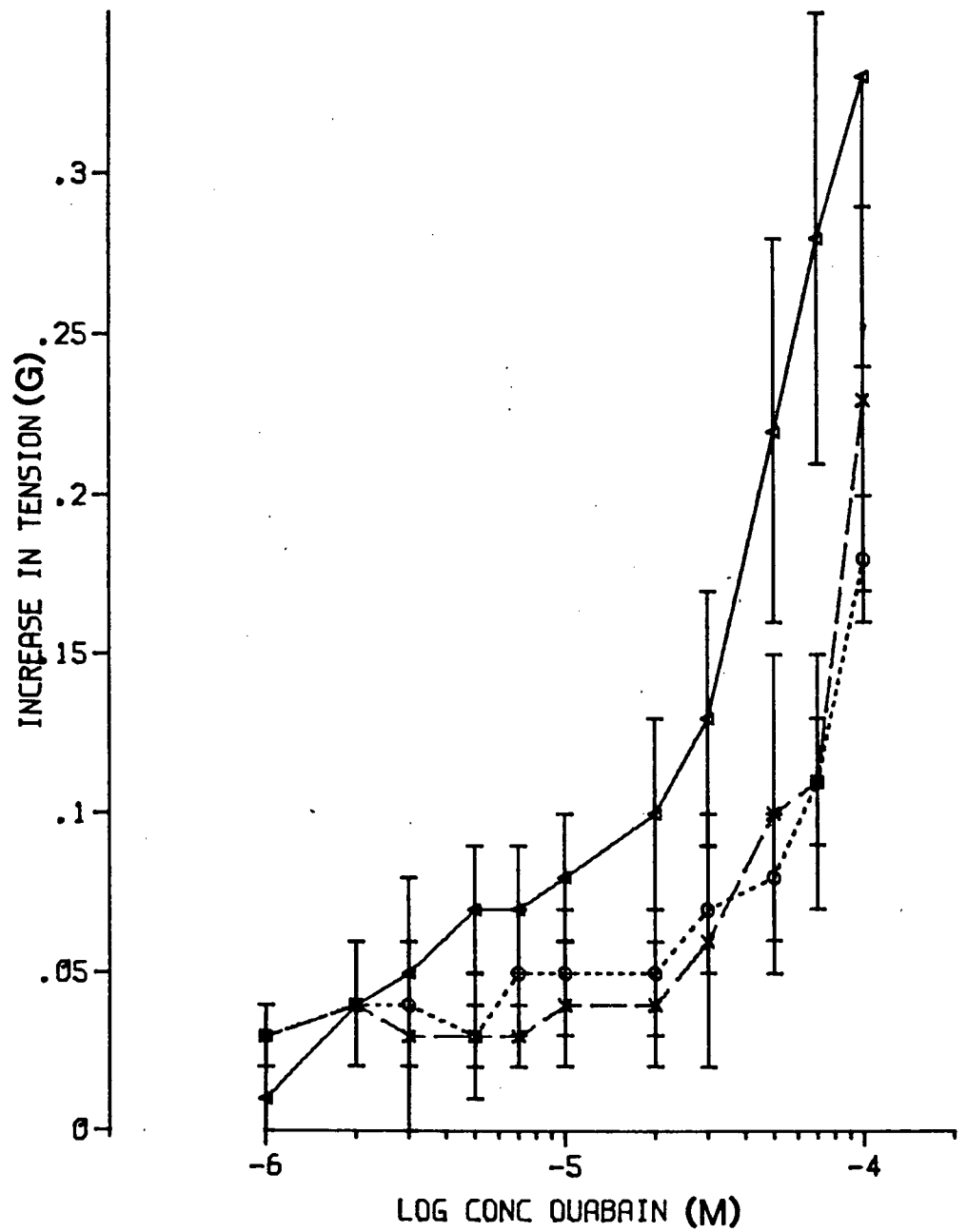(○) STZ (N=6)
(x) ALLOXAN (N=4)

FIG 9

Fig 10

Inotropic Response of Left Atria from Control and 6 Month
Diabetic Rats to Ouabain.

Tissues were stimulated with square wave pulses at a frequency
of 3.3 hz and equilibrated, at 37°, for 1 hour in oxygenated
CK buffer, pH 7.4, prior to drug addition. Dose response
curves were performed in a cumulative manner.

Response is expressed in terms of increase in developed tension
(g) vs. the log of the molar concentration of ouabain.   Each
point represents the mean ± SEM. $^{\Delta}$Control n=9; ^{X}Alloxan n=6;
^{O}STZ n=4.

*p<.05 compared to control.

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RESPONSE TO OUABAIN
(◄) CONTROL (N=9)
(×) ALLOXAN (N=6)
(o) STZ (N=4)

FIG 10

Fig 11

Inotropic Response of Papillary Muscles from Control and
7 Day Diabetic Rats to Ouabain.

Tissues were stimulated with square wave pulses at a frequency
of 1 hz and were equilibrated, at 37°, for 1 hour in oxygenated
CK buffer, pH 7.4. Isoproterenol dose response curves were
then performed, tissues were washed for a period of 1 hour and
ouabain dose response curves were performed in a cumulative
manner.

Response is expressed in terms of increase in tension (g) vs.
the log of the molar concentration of ouabain. Each point
represents the mean ± SEM. ΔControl n=15; $\circ$STZ n=8.

RESPONSE TO OUABAIN
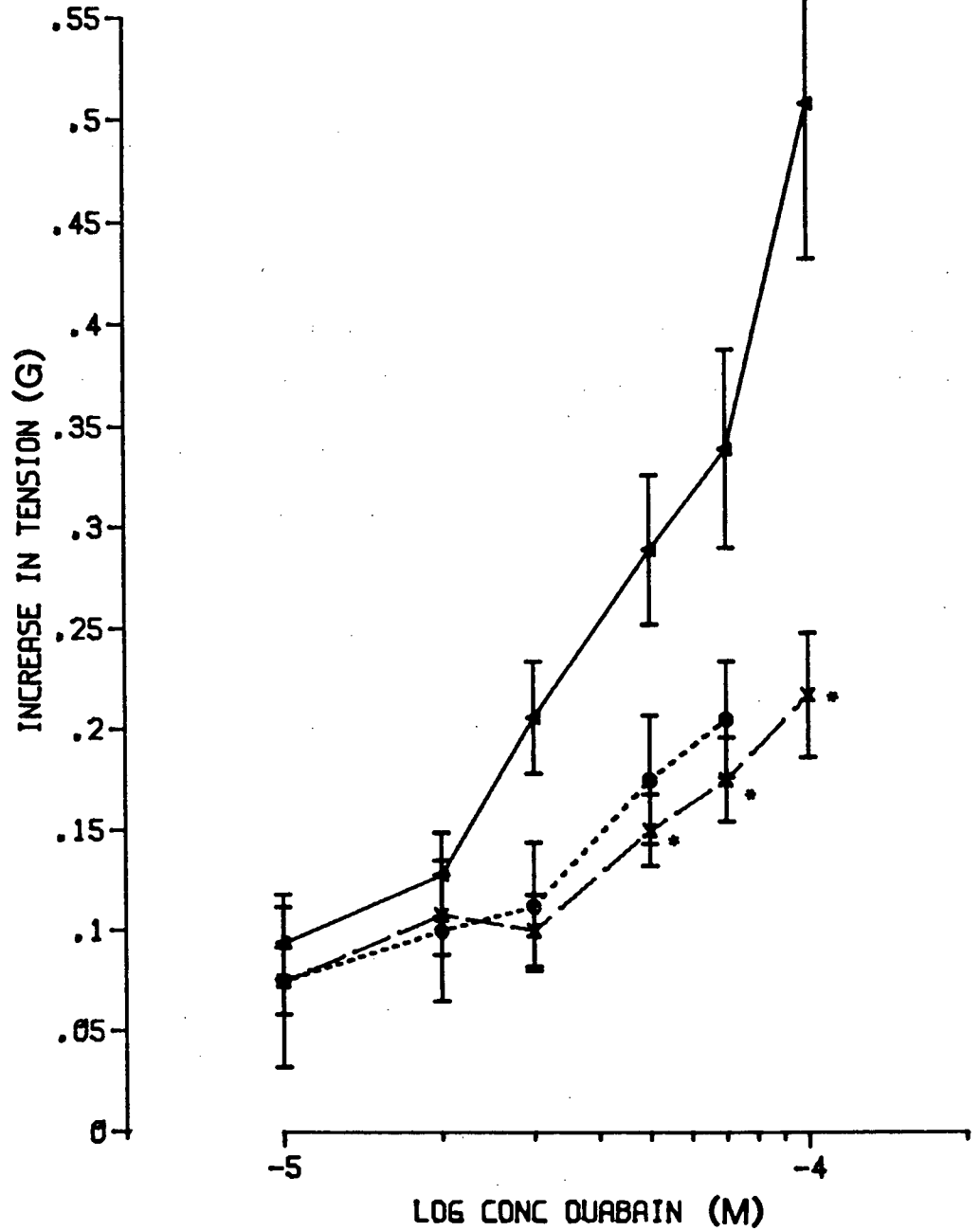(◄) CONTROL (N=15)
(○) STZ (N=8)
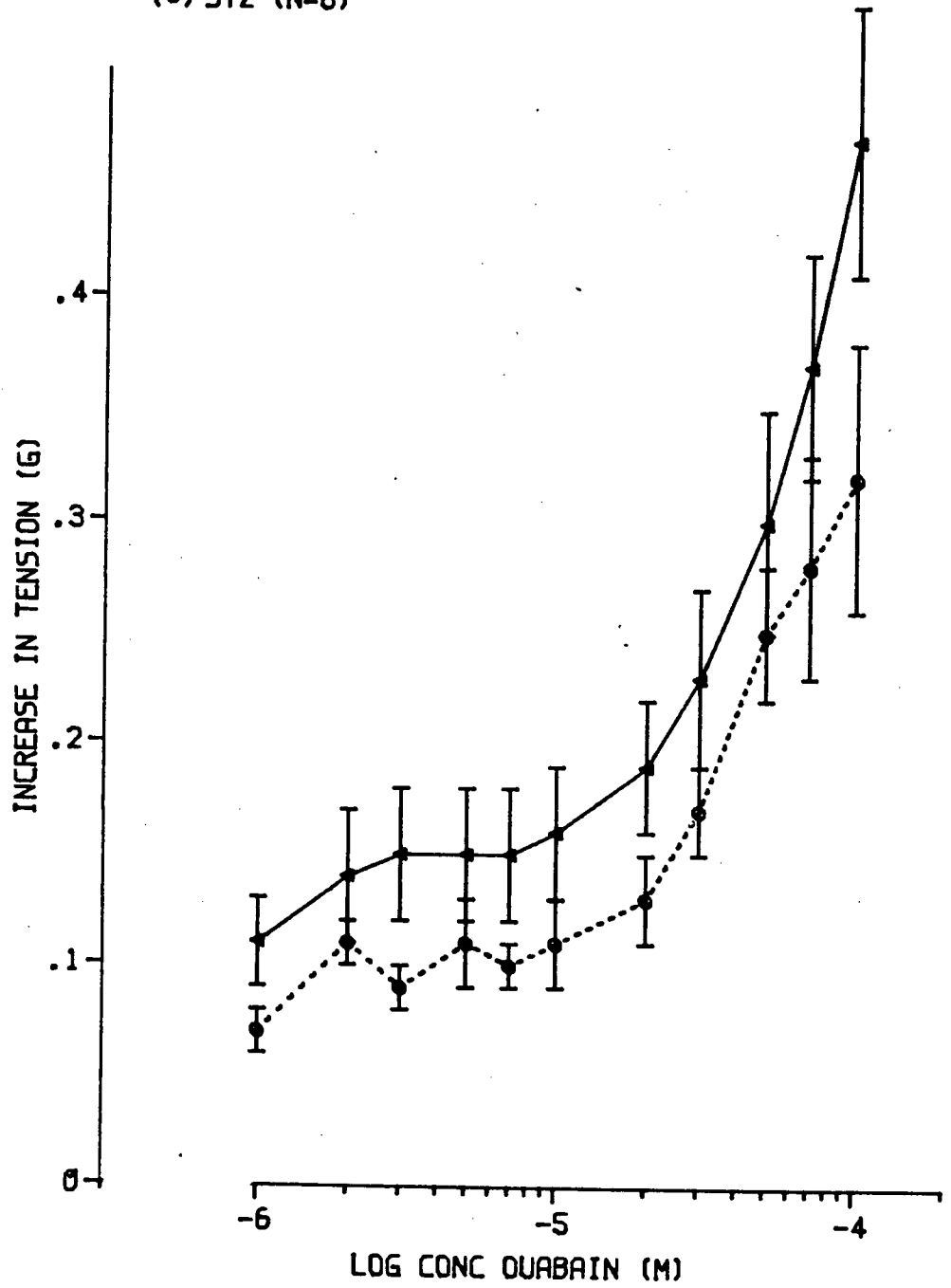
FIG 11

Fig 12

Inotropic Response of Papillary Muscles from Control and
70 Day Diabetic Rats to Ouabain.

Tissues were stimulated with square wave pulses at a
frequency of 1 hz and equilibrated, at 37°, for 1 hour
in oxygenated CK buffer, pH 7.4. Isoproterenol dose
response curves were performed, tissues were washed for
a period of 1 hour and then ouabain dose response curves
were obtained. Dose response curves were performed in a
cumulative manner.

Response is expressed in terms of increase in tension vs.
the log of the molar concentration of ouabain. Each point
represents the mean ± SEM. $^{\Delta}$Control n=5; ^{O}STZ n=12.

*p<.05 compared to control.

RESPONSE TO OUABAIN
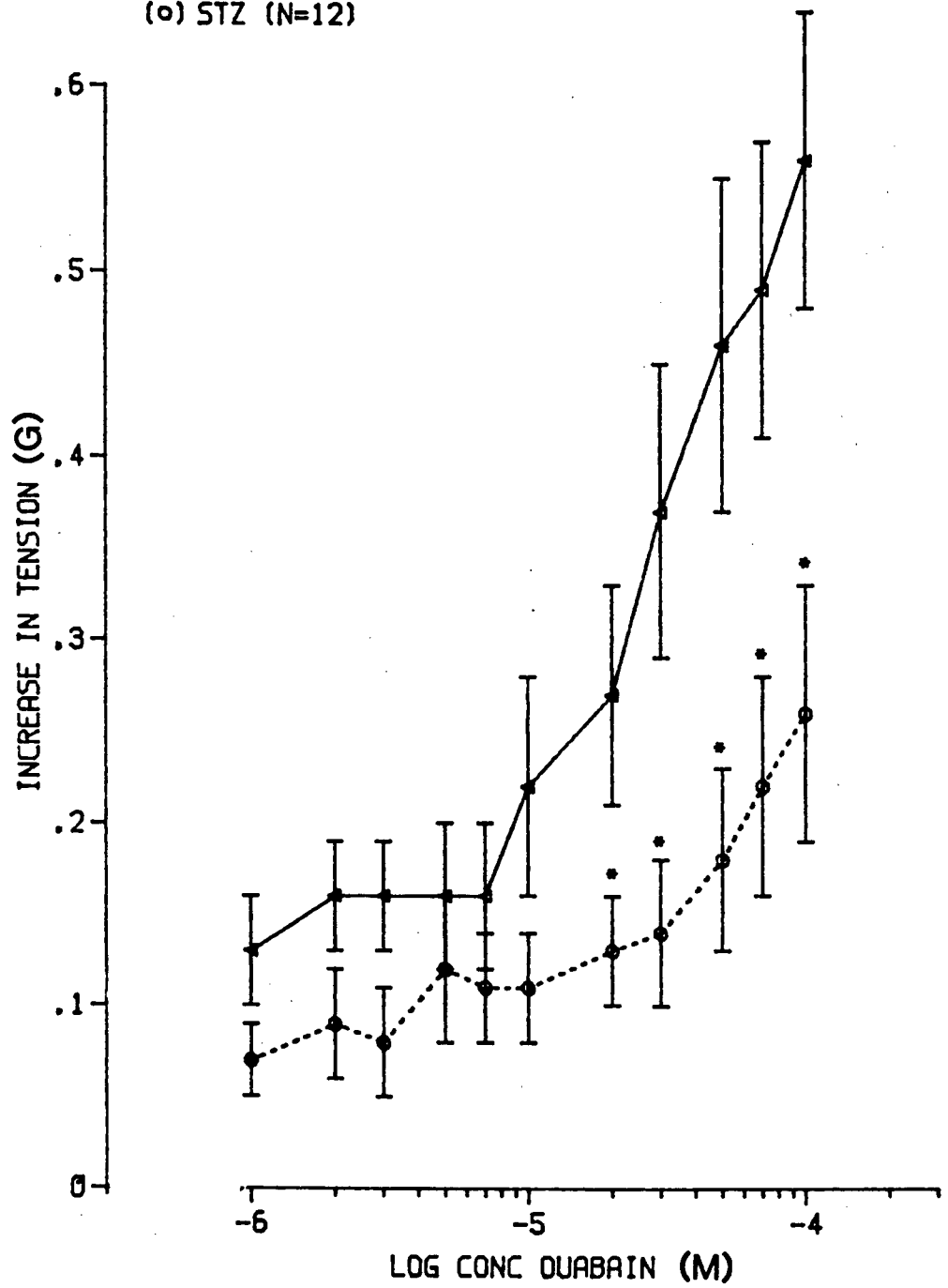(◄) CONTROL (N=5)
(o) STZ (N=12)

FIG 12

curves to ouabain were then obtained. As previously observed, β blockade did not reduce the positive inotropic effect of ouabain in left atria (Fig 13). These tissues demonstrated virtually no increase in tension until doses of ouabain greater than 10^{-5} M were administered. There was no significant difference in the curves obtained from tissues which were equilibrated for 1 hour vs. those equilibrated for 3 hours.

The dose response curves obtained from papillary muscles were very different from those of atria (Fig 14). Administration of ouabain to left atria produced a monophasic dose response curve while papillary muscles responded in a biphasic manner. There was an initial increase in tension when ouabain was administered in dose of 10^{-7} to 10^{-6} M which accounted for less than 50% of the total increase in tension. Administration of similar concentrations of ouabain had no effect on tension development in atrial tissues. (Fig 13). There was very little change in tension when concentrations of 10^{-6} to 10^{-5} M were added. The greatest increase in tension was observed when ouabain was administered to papillary muscles in doses of 10^{-5} to 10^{-4} M.

Timolol did not block the response of papillary muscles to any dose of ouabain. The other variable which was examined was time and it appeared to play an important role in the response of the tissues. Papillary muscles which had been equilibrated for three hours demonstrated a significantly greater response to ouabain at most points throughout the dose response curve than did tissues which had been equilibrated for only one hour.

Fig 13

Inotropic Response of Left Atria to Ouabain.

Response is expressed in terms of increase in tension (g) vs. the log of the molar concentration of ouabain. Each point represents the mean ± SEM. $\triangle$1 hour equilibration in normal buffer n=4; ^{X}1 hour equilibration in buffer containing 10^{-7}M timolol n=4; $\square$3 hour equilibration in normal buffer n = 4; $^+$3 hour equilibration in buffer containing 10^{-7}M timolol n=4. In each case, cumulative dose response curves followed the equilibration period.

RESPONSE TO OUABAIN
(◄) 1 HR CK (N=4)
(×) 1 HR CK + TIM (N=4)
(□) 3 HR CK (N=4)
(+) 3 HR CK + TIM (N=4)
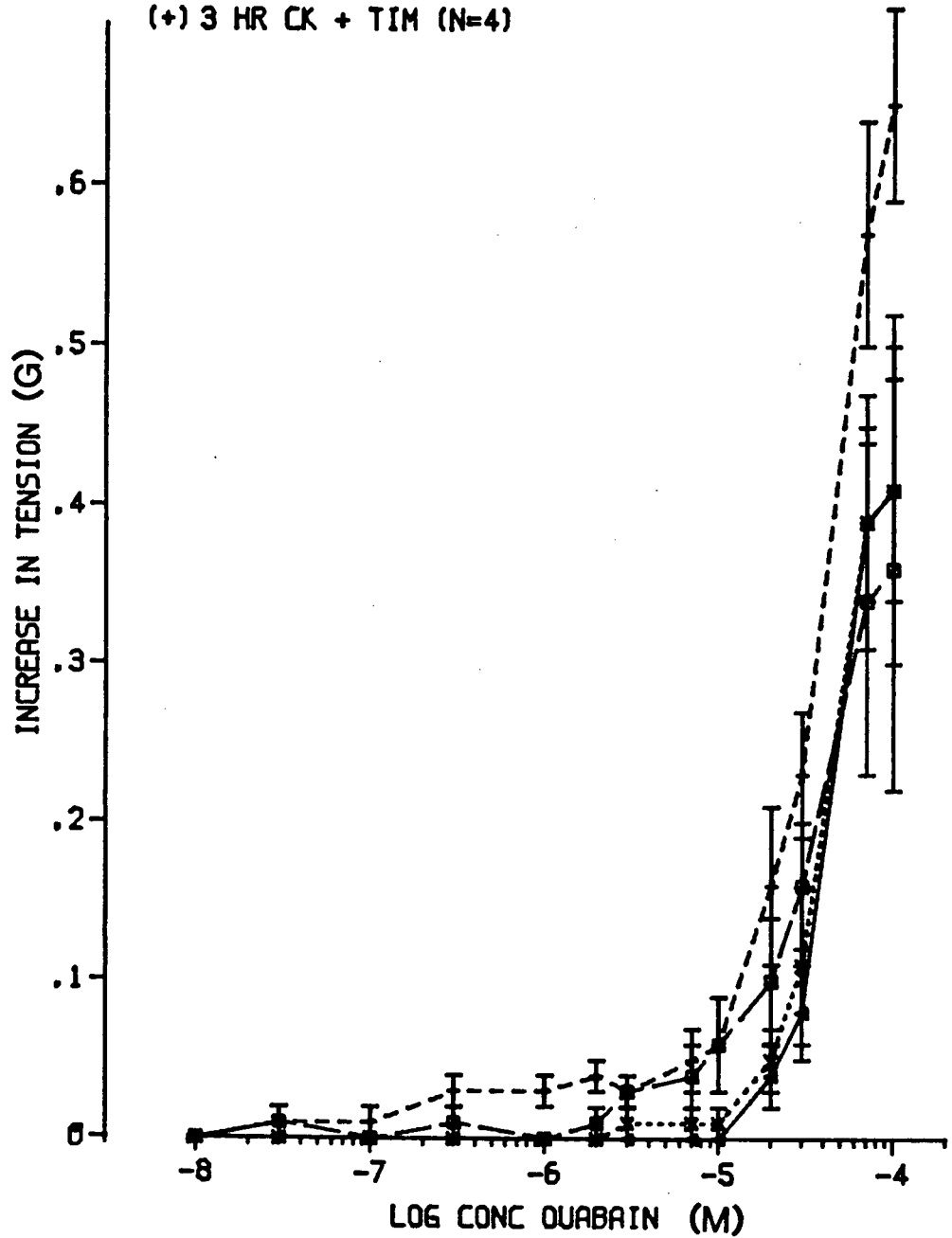
FIG 13

Fig 14

Inotropic Response of Papillary Muscles to Ouabain.

Response is expressed in terms of increase in tension (g)

vs. the log of the molar concentration of ouabain. Each

point represents the mean ± SEM. $^{\Delta}$1 hour equilibration

in normal buffer n=4; ^{X}1 hour equilibration in buffer

containing 10^{-7}M timolol n=8; $^{\square}$3 hour equilibration in

normal buffer n=8; †3 hour equilibration in buffer

containing 10^{-7}M timolol. In each case, cumulative dose

response curves followed the equilibration.

*p<.05 compared to Δ

**p<.05 compared to Δ and X

***p<.05 compared to Δ, X and $\square$

RESPONSE TO OUABAIN
(◄) 1 HR, CK (N=4)
(✗) 1 HR, CK + TIM (N=8)
(□) 3 HR, CK (N=8)
(+) 3 HR, CK + TIM (N=4)

FIG 14

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DISCUSSION

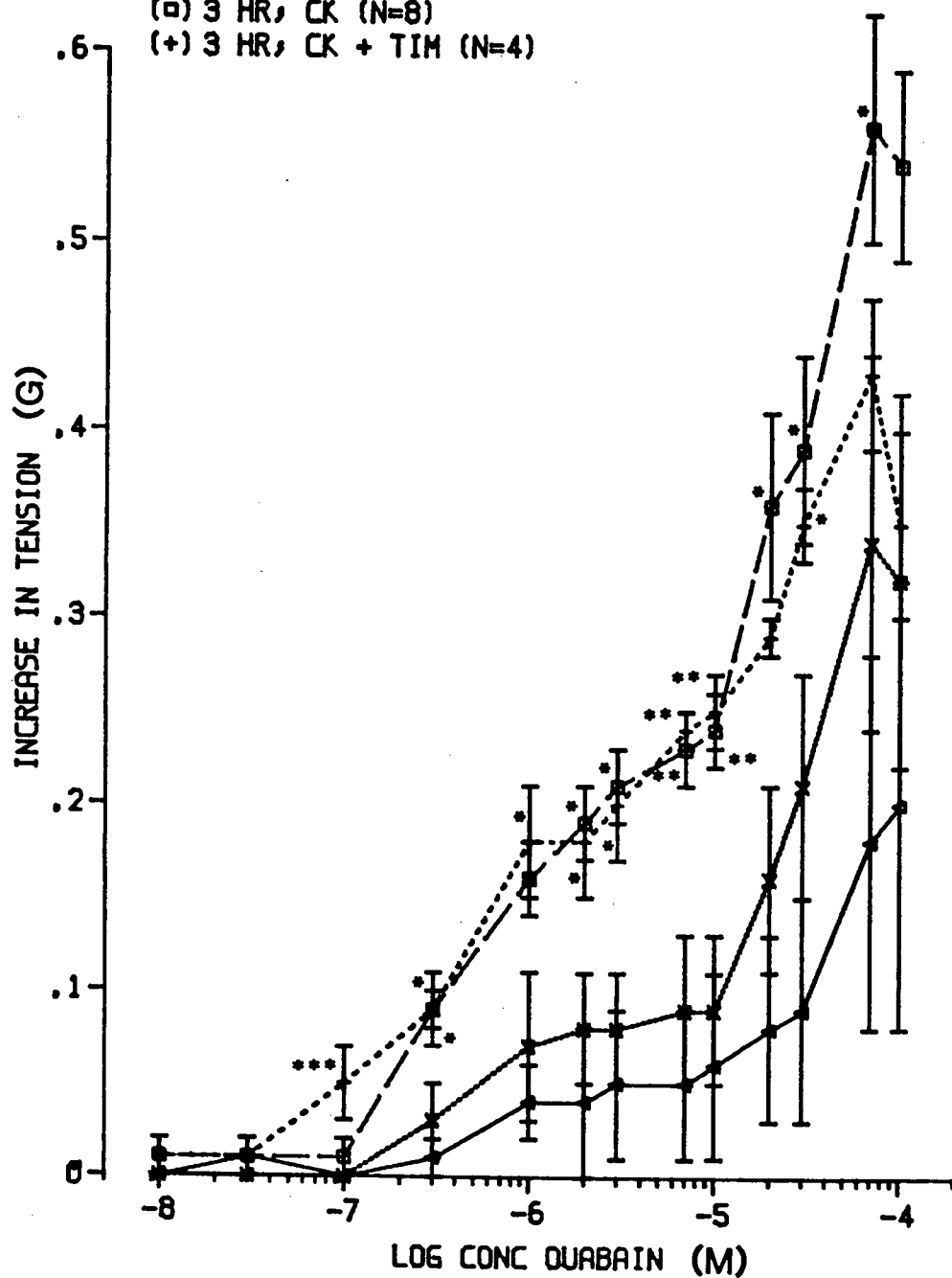### I. Inotropic Response to d,l Isoproterenol

The data presented here show no significant differences in
the positive inotropic responses to isoproterenol in isolated cardiac
tissues from 7 or 70 day diabetic rats as compared to controls (Fig 2-5).
A tendency toward a depression of the responses was evident in
papillary muscles and to a lesser extent in left atria from 70 day
diabetic animals (Fig 3 and 5). Failure to observe a statistically
significant difference may be due to the use of a relatively small
number of tissues or to a very small difference in the two populations.
Our hypothesis would suggest that changes might become more evident
at a later time point due to their progressive nature.

Although we have failed to demonstrate a statistically signif-
icant change, the observed depression is consistent throughout the
curves and is worthy of comment. The depression reported here is in
agreement with the observations of Foy and Lucas (1976) of a
decreased sensitivity to isoproterenol in hearts from diabetic rats.
Also in agreement are the observations of Vadlamudi and McNeill (1981a)
who reported a depression of the relaxant effect of isoproterenol
at several time points following the induction of diabetes.

If the depression observed represents a real change within the
population, or even if such a change was demonstrated at a later
time point, the molecular basis for such an occurance remains unknown.
Savarese and Berkowitz (1979) reported a 28% decrease in the number of
β adrenoreceptors in cardiac tissue of 2 month STZ diabetic rats.

In 1956 Stephenson introduced the concept of spare receptors. The small decrease in receptor number reported by Savarese and Berkowitz would not be expected to alter the magnitude of the β adrenoreceptor mediated response.

A second site at which the response to isoproterenol could be modulated is the cAMP, protein kinase cascade system. Ingebretson et al. (1981) reported a depression in isoproterenol induced changes in cAMP and protein kinase and no change in phosphorylase activation in hearts from diabetic animals while Miller et al., (1981) and Vadlamudi and McNeill (1982) reported enhanced phosphorylase activation in hearts from diabetic rats. The reason for the discrepancy in these results is not immediately evident. The most notable difference in the studies is that Ingebretson et al.,(1981) administered insulin to their animals up until 4 days prior to sacrifice. Regarding the possibility of enhanced phosphorylase activation, the role of calcium in this condition has not been fully defined. It is possible that calcium is the mediator of the enhanced activation.

II. Chronotropic Response to d,l Isoproterenol

The chronotropic response to isoproterenol parallels the inotropic one. Seven days after the induction of diabetes, there was no difference in the response of right atria from diabetic animals as compared to controls (Fig 6). Seventy days after the induction of diabetes, a non significant depression was evident throughout the dose response

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curve (Fig 7). As noted above, failure to demonstrate a statistically significant difference may be due to the small sample size or to a very small difference within the population.

If the observed trend represents a real change one would expect that other responses, mediated by the same receptor and post receptor events, would be altered in a similar manner. As reported here, seventy days after the induction of diabetes papillary muscles and, to a lesser extent, left atria demonstrated slightly depressed inotropic responses to isoproterenol and right atria display slightly depressed chronotropic responses to this drug.

The molecular basis for a possible depression of the chronotropic response is not fully understood however the possibilities of an alteration at the β receptor level or at post receptor events are discussed in the section immediately preceeding this (I. Inotropic Responses to d,l Isoproterenol).

III. Response to Ouabain

Papillary muscles appeared to have a biphasic response to ouabain (Fig 11 and 12). Low doses ($<10^{-6}$M) evoked a small increase in tension however the principal inotropic event occured when ouabain was administered in concentrations of 10^{-5} to 10^{-4}M. Left atria responded in a monophasic manner to ouabain concentrations of 10^{-5} to 10^{-4}M (Fig 9 and 10).

The observation of a nonsignificant depression of ouabain responses in 7 day diabetic tissues (Fig 9 and 11) is consistent with our hypothesis of the time dependence of the cardiomyopathy which develops with diabetes. It is interesting that at this time point, no trend was evident regarding the inotropic response to isoproterenol (Fig 2 and 4).

The depression of the response to ouabain was more evident at later time points. A statistically significant difference was observed in papillary muscles from 70 day diabetic rats (Fig 12). Although the low-dose response was inhibited, the depression was small and not statistically significant. The major effect of ouabain was on the high-dose response. In left atria from 6 month diabetic animals the maximum inotropic response to ouabain was also inhibited (Fig 10). These observations are in agreement with those of Sellers and Ku (1981) who reported a decrease in the maximum inotropic response to ouabain of left atria from diabetic rats.

The data presented here provide very little insight into the molecular mechanism responsible for this depression however the major influence of diabetes appears to be on the high-dose effect of ouabain in papillary muscles and the response in left atria. Although there is some confusion in the literature regarding the nature of the low-dose effect, it appears that the high-dose effect in papillary muscles and the response of left atria are mediated through inhibition of $(Na^+ + K^+)$-ATPase (Schwartz et al., 1981). It appears that diabetes alters the $(Na^+ + K^+)$-ATPase mediated positive inotropic effect of cardiac glycosides. Ku (1980) reported a decrease in sodium pump activity in hearts of 5 week diabetic rats. The levels of long chain acyl carnitines are elevated in the hearts of diabetic animals (Shug et al. 1975), and palmityl carnitine, the most abundant member of this group has been shown to inhibit $(Na^+ + K^+)$-ATPase. Both of these observations suggest that a decrease in $(Na^+ + K^+)$-ATPase activity may occur in the diabetic heart. Ouabain would then have less enzyme units available for inhibition in the diabetic

heart as compared to control and would have a lesser effect on the diabetic heart. The data presented here support such a theory: tissues from diabetic animals displayed smaller inotropic responses to ouabain than did tissues from control animals.

There has been very little investigation into the activity of $(Na^+ + K^+)$-ATPase in the diabetic heart however Onji and Liu (1980) reported that there was no difference in the number of ouabain binding sites in hearts of 5 to 8 day alloxan diabetic dogs. It would be interesting to repeat this experiment at a later time point following the induction of diabetes. It would also be of interest to measure the affinity of ouabain for the binding sites in diabetic and control animals.

Lindemeyer and Wellsmith (1980) reported two conformations of $(Na^+ + K^+)$-ATPase. Ouabain was bound to both enzyme conformations however only one conformation was involved in the production of the inotropic response. It would be interesting to investigate the influence of diabetes on the conformation of $(Na^+ + K^+)$-ATPase.

Although the majority of evidence suggests that inhibition of the sodium pump is responsible for at least the major component of the cardiac glycoside induced positive inotropic response, the possibility remains that these two events are not causally linked and that the effect of diabetes is at a site other than $(Na^+ + K^+)$-ATPase.

IV. __Effect of Time and β Blockade on the Ouabain Dose Response Curve__

The literature provides conflicting evidence regarding the

inotropic response of cardiac tissues to low concentrations of cardiac glycosides. The data presented in this study indicate that a possible reason for the controversy is that ouabain produces a monophasic response in left atria and a biphasic response in papillary muscles from rats (Fig 13 and 14). These observations are in agreement with those of Schwarts et al. (1981) who reported a monophasic response in rat left atria and a biphasic response in right ventricular strips with a low-dose (ED_{50} of 3×10^{-7}M) and a high-dose (ED_{50} of 3.5×10^{-5}M). Although we were unable to calculate accurate ED_{50} values due to the steepness of the curves it is evident in Fig 14 that the response which we observed in papillary muscles was very similar to that found by Schwartz et al. Similar results were reported by Grupp et al. (1981).

Timolol, a β adrenergic antagonist, had no effect on the response of papillary muscles or atria to ouabain. Schwartz et al. (1981) and Grupp et al. (1981) made similar observations. Grupp et al. (1981) reported that a second dose response curve in papillary muscles displayed only a single component and suggested that this was a result of desensitization. During the washout period the tension did not fall to predrug levels and it is possible that the investigators' failure to observe the low-dose effects was due to the fact that the effect was not fully washed out and was still present when the second dose response curve was performed. Schwartz et al. (1981) proposed that the high affinity response was mediated by the action of ouabain on a site unrelated to ($Na^+ + K^+$)-ATPase and that binding to this site resulted in a direct increase in the intracellular calcium concentration. They suggested that the low

affinity site in ventricle (and the site in atria) was $(Na^+ + K^+)$-ATPase and that ouabain binding inhibited the enzyme and eventually led to an increase in intracellular calcium. The final common step in both mechanisms of inotropy was the increase in intracellular calcium which would then be available to interact with the contractile elements. At this time it appears that there are two ouabain binding sites in rat ventricular tissue. It is not clear whether both of these sites are related to the sodium pump. Equally unclear is the role of this second site in tissues displaying a monophasic response to ouabain. Schwartz et al. (1981) suggested that this site may be present in all tissues with its affinity being very similar to that of the site on $(Na^+ + K^+)$-ATPase in tissues displaying a monophasic response to cardiac glycosides. At this time the identity of this site as well as its distribution remains unclear.

The other variable which we examined was time. It has been reported that the response of isolated cardiac tissues to ouabain is dependent on the period of equilibration. Carrier et al. (1974) reported that the response of guinea pig atria to ouabain was enhanced in tissues equilibrated for five hours compared to those equilibrated for only one hour. The authors actually observed a decrease in systolic tension during the longer equilibration period and although ouabain caused a greater increase in tension in these tissues the maximum tension developed did not appear to be different from that developed by tissues equilibrated for only one hour. We did not observe any difference in predrug systolic tensions in atria or papillary muscles equilibrated for one or three hours. The response of papillary muscles to cardiac glycosides in the present studies was

enhanced by long periods of equilibration. Care should therefore be taken to employ the same equilibration time prior to all ouabain dose response curves. The increased equilibration period had a much less dramatic effect on the response of atria to ouabain. It is possible that the papillary muscles became slightly hypoxic over the long equilibration period despite oxygenation of the buffer.

V. Conclusions

1. Statistically significant differences in inotropic and chronotropic responses to d,l isoproterenol could not be detected in isolated cardiac tissues from 7 or 70 day diabetic rats as compared to controls. A trend toward a decrease in inotropic and chronotropic response was observed in tissues 70 days after the induction of diabetes.

2. Isolated cardiac tissues from chronically diabetic rats had a decreased capacity to respond to cardiac glycosides. The effect was not evident in tissues from acutely diabetic (7 day) rats.

3. Ouabain produced a monophasic response in left atria and a biphasic response in left papillary muscles. The response was not mediated by catecholamine release.

4. The response of papillary muscles to ouabain was enhanced by increasing equilibration periods while that of left atria was not significantly altered.

Adams, R.J., Cohen, D.W., Gupte, S., Johnson, J.D., Wallick, E.T., Wang, T. and Schwartz, A.: In vitro effects of palmitylcarnitine on cardiac plasma membrane Na, K-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase and Ca^{2+} transport. J. Biol. Chem. <u>254</u>: 12404-12410, 1979.

Ahmed, S.S., Jaferi, G.A., Narang, R.M., and Regan, T.J.: Preclinical abnormality of left ventricular function in diabetes mellitus. Am. Heart J. <u>89</u>: 153-158, 1975.

Akera, T., Baskin, S.I., Tobin, T. and Brody, T.M.: Ouabain: temporal relationship between the inotropic effect and the in vivo binding to and dissociation from Na+K activated ATPase. Naunyn-Schmideberg's Arch. Pharmacol. <u>277</u>: 151-162, 1973.

Akera, T. and Brody, T.M.: The role of Na^+, K^+-ATPase in the inotropic action of digitalis. Pharmacol. Rev. <u>29</u>: 187-220, 1978.

Akera, T., Ku, D. and Brody, T.M.: Alterations of ion movements as a mechanism of drug induced arrhythmias and inotropic responses. In Taurine, ed. by R. Huxtable and A. Barbeau, pp. 121-134, Raven Press, New York, 1975.

Akera, T., Yamamoto, S., Chubb, J., McNish, R. and Brody, T.M.: Biochemical basis for the low sensitivity of the rat heart to digitalis. Naunyn-Schmiedeberg's Arch. Pharmacol. <u>308</u>: 81-88, 1979.

Alavaikko, M., Elfring, R., Hirvonen, J. and Jarvi, J.: Triglycerides, cholesterol, and phospholipids in normal heart papillary muscle and in patients suffering from diabetes, choleslithiasis, hypertension and coronary atheroma. J. Clin. Path. <u>26</u>: 283-293, 1973.

Allen, J.C. and Schwartz, A.: A possible biochemical explanation for the insensitivity of the rat to cardiac glycosides. J. Pharmacol. Exp. Ther. <u>168</u>: 42-46, 1969.

Baandrup, U., Ledet, T. and Rasch, R.: Experimental diabetic cardiopathy preventable by insulin treatment. Lab. Invest. <u>45</u>: 169-173, 1981.

Baily, L.E. and Dresel, P.E.: Role of the sugar transport system in the positive response to digitalis. J. Pharmacol. Exp. Ther. <u>176</u>: 538-543, 1971.

Banting, F.G. and Best, C.H.: The internal secretion of the pancreas. J. Lab. Clin. Med. <u>7</u>: 251-266, 1922.

Banting, F.G., Best, C.H., Collip, J.B., Campbell, W.R. and Fletcher, A.A.: Pancreatic extracts in the treatment of diabetes mellitus. Can. Med. Assoc. J. <u>12</u>: 141-146, 1922.

Benforando, J.M.: Frequency-dependent pharmacological and physiological effects on the rat ventricle strip. J. Pharmacol. Exp. Ther. <u>122</u>: 86-100, 1958.

Bentfield, M., Lullmann, H., Peters, T. and Proppe, D.: Interdependence of ion transport and the action of ouabain in heart muscle. Br. J. Pharmacol. 61: 19-27, 1977.

Bergstrand, A. and Bucht, H.: Electron microscopic investigations on the glomerular lesions in diabetes mellitus (diabetic glomerulosclerosis). Lab. Invest. 6: 293-300, 1957.

Blesa, E.S., Langer, G.A., Brady, A.J. and Serena, S.D.: Potassium exchange in rat ventricular myocardium: its relation to rate of stimulation. Am. J. Physiol. 219: 747-754, 1970.

Boucher, C.A., Fallon, J.T., Johnson, R.A. and Yurchak, P.M.: Cardiomyo-pathic syndrome caused by coronary artery disease III: prospective clinicopathological study of its prevalence among patients with clinically unexplained chronic heart failure. Br. Heart. J. 41: 613-620, 1979.

Carrier, G.O., Lullmann, H., Neubauer, L. and Peters, T.: The significance of a fast exchanging superficial calcium fraction for the regulation of contractile force in heart muscle. J. Mol. Cell. Cardiol. 6: 333-347, 1974.

Chenoweth, M.B. and Koelle, E.S.: An isolated heart perfusion system adapted to the deterination of non-gaseous metabolites. J. Lab. Clin. Med. 31: 600-608, 1946.

Das, I.: Effect of diabetes and insulin on the rat heart adenyl cyclase, cyclic AMP phosphodiesterase and cyclic AMP. Horm. Metab. Res. 5: 330-333, 1973.

Denton, R.M. and RAndle, P.J.: Concentrations of glycerides and phospho-lipids in rat heart and gastrocnemius muscles: effects of alloxan-diabetes and perfusion. Biochem. J. 104: 416-422, 1967.

Dillman, W.H.: Diabetes mellitus induces changes in cardiac myosin of the rat. Diabetes 29: 579-582, 1980.

Dunn, J.S., Kirkpatrick, J., McLetchie, N.G.B. and Telfers, S.V.: Necrosis of islets of Langerhans produced experimentally. J. Path. Bacteriol. 55: 245-252, 1943.

Erdmann, E., Brown, L., Krawietz, W. and Werdan, K.: Quantitative evaluation of ^{3}H-ouabain binding to contracting heart muscle, positive inotropy, Na/K-ATPase inhibition and ^{86}Rb$^+$ - uptake in several species. Ab. Third. Ann. Inter. Conf. Na,K-ATPase 3: 19, 1981.

Erdmann, E., Philip, G. and Scholz, H.: Cardiac glycoside receptor, (Na$^+$ + K$^+$)-ATPase activity and force of contraction in rat heart. Biochem. Pharmacol. 29: 3219-3229, 1980.

Fein, F.S., Kornstein, L.B., Strobeck, J.E., Capasso, J.M. and Sonnenblick, E.H.: Altered myocardial mechanics in diabetic rats. Circ. Res. 47: 922-933, 1980.

Fein, F.S., Strobeck, J.E., Malhotra, A., Scheuer, J. and Sonnenblick, E.H.: Reversibility of diabetic cardiomyopathy with insulin in rats. Circ. Res. 49: 1251-1261, 1981.

Feuvray, D., Idell-Wenger, J.A. and Neely, J.R.: Effects of ischemia on rat myocardial function and metabolism in diabetes. Circ. Res. 44: 322-329, 1979.

Foy, J.M. and Lucas, P.D.: Effect of experimental diabetes, food deprivation and genetic obesity on the sensitivity of pithed rats to autonomic agents. Br. J. Pharmacol. 57: 229-234, 1976.

Friedenwald, I.S.: Diabetic retinopathy. Am. J. Ophthalmol. 33: 1187-1199, 1950.

Ganda, O.P., Rossini, A.A. and Like, A.A.: Studies on streptozotocin diabetes. Diabetes 25: 595-603, 1976.

Ghysel-Burton, J. and Godfraind, T.: Stimulation and inhibition of the sodium pump by cardioactive steroids in relation to their binding sites and their inotropic effect on guinea pig isolated atria. Br. J. Pharmacol. 66: 175-184, 1979.

Glynn, I.M. and Karlish, S.J.D.: The sodium pump. Ann. Rev. Physiol. 37: 13-55, 1975.

Godfraind, T., DePover, A. and Lutete, D.T.: Identification with potassium and vanadate of two classes of specific ouabain binding sites in a ($Na^+ + K^+$)-ATPase preparation from the guinea pig heart. Biochem. Pharmacol. 29: 1195-1199, 1980.

Godfraind, T. and Ghysel-Burton, J.: Binding sites related to ouabain-induced stimulation or inhibition of the sodium pump. Nature (London) 265: 165-166, 1977.

Godfraind, T. and Ghysel-Burton, J.: Independence of the positive inotropic effect of ouabain from the inhibition of the heart Na^+/K^+ pump. Proc. Natl. Acad. Sci. 77: 3067-3069, 1980.

Grupp, G., Grupp, I.L., Ghysel-Burton, J., Godfraind, T. and Schwartz, A.: Effects of very low concentrations of ouabain on contractile force of isolated guinea pig, rabbit and cat atria and right ventricular papillary muscles: an interinstitutional study. J. Pharmacol. Exp. Ther. 220: 145-151, 1982.

Grupp, I.L., Grupp, G. and Schwartz, A.: Digitalis receptor desensitization in rat ventricle: ouabain produces two inotropic effects. Life Sci. 29: 2789-2794, 1981.

Haider, B., Yen, C.K., Thomas, G., Oldewurtel, H.A., Lyons, M.M. and Regan, T.J.: Influence of diabetes on the myocardium and coronary arteries of rhesus monkey fed an atherogenic diet. Circ. Res. 49: 1278-1288, 1981.

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Hearse, D.J., Stewart, D.A. and Chain, E.B.: Diabetes and the survival and recovery of the anoxic myocardium. J. Mol. Cell. Cardiol. <u>7</u>: 397-415, 1975.

Hougan, T.J. and Smith, T.W.: Inhibition of myocardial monovalent cation active transport by subtoxic doses of ouabain in the dog. Circ. Res. <u>42</u>: 856-863, 1978.

Hougan, T.J. and Smith, T.W.: Biphasic effect of cardiac glycosides on the sodium pump: role of catecholamines. Circulation <u>62</u>: Supp. III 987, 1980.

Ingebretson, C.G., Moreau, P., Hawelu-Johnson, C. and Ingebretson, W.R.: Performance of diabetic rat hearts: effects of anoxia and increased work. Am. J. Physiol. <u>239</u>: H614-H620, 1980.

Ingebretson, W.R., Peralta, C., Monsher, M., Wagner, L.K. and Ingebretson, C.G.: Diabetes alters the myocardial cAMP-protein kinase cascade system. Am. J. Physiol. 240: H375-H383, 1981.

Junod, A., Lambert, A.E., Orci, L., Piclet, R., Gonet, A.E. and Renold, A.E.: Studies on the diabetogenic action of streptozotocin. Proc. Soc. Exp. Biol. Med. <u>126</u>: 201-205, 1967.

Kannel, W.B., Hjortland, M. and Castelli, W.P.: Role of diabetes in congestive heart failure. Am. J. Cardiol. <u>34</u>: 29-34, 1974.

Katzung, B., Rosin, H. and Scheider, F.: Frequency-force relationship in the rabbit auricle and its modification by some metabolic inhibitors. J. Pharmacol. <u>120</u>: 324-333, 1957.

Kaul, C.L. and Grewal, R.S.: Increased urinary excretion of catecholamines and their metabolites in streptozotocin diabetic rats. Pharmacol. 21: 223-228, 1980.

Kerby, A.L., Randle, P.J., Cooper, R.H., Whitehouse, S., Pask, H.T. and Denton, R.M.: Regulation of pyruvate dehydrogenase in rat heart. Biochem. J. <u>154</u>: 327-348, 1976.

Kruta, V.: Sur l'activite rhythmique du muscle cardiaque. I. Variations de la response mechanique en fonction du rythme. Arch. Int. Physiol. <u>45</u>: 332-357, 1937.

Ku, D.D.: Decreased sodium pump activity in heart and thoracic aorta of streptozotocin-induced diabetic rats. The Pharmacologist. <u>22</u>: 288, 1980.

Ku, D., Akera, T., Tobin, P. and Brody, T.M.: Comparative species studies on the effect of monovalent cations and ouabain on cardiac Na^+ K^+ adenosine triphosphatase and contractile force. J. Pharmacol. Exp. Ther. <u>197</u>: 458-469, 1976.

Langer, G.A.: Kinetic studies of calcium distribution in ventricular muscle of the dog. Circ. Res. 15: 393-405, 1964.

Langer, G.A.: The role of sodium ion in the regulation of myocardial contractility. J. Mol. Cell. Cardiol. 1: 203-207, 1970.

Langer, G.A. and Serena, S.D.: Effects of strophanthidin upon contraction and ionic exchange in rabbit ventricular myocardium: relation to control of active state. J. Mol. Cell. Cardiol. 1: 65-90, 1970.

Ledet, T., Neubauer, B., Christensen, N.J. and Lundbaek, K.: Diabetic cardiopathy. Diabetologia, 16: 207-209, 1979.

Lopaschuk, G.D., McNeill, J.H. and Katz, S.: Effect of diabetes on Ca^{++}-transport in cardiac sarcoplasmic reticulum. The Pharmacologist 23: 198, 1981.

Malhotra, A., Penpargkul, S., Fein, F.S., Sonnenblick, E.H. and Scheuer, J.: The effect of streptozotocin-induced diabetes in rats on cardiac contractile proteins. Circ. Res. 49: 1243-1250, 1981.

Marble, A.: Insulin-clinical aspects: the first fifty years. Diabetes 21: Supp. 2, 632-636, 1972.

McCans, J.L., Lindenmeyer, G.E., Munson, R.G., Evans, R.W. and Schwartz, A.: A dissociation of positive staircase (Bowditch) from ouabain-induced positive inotropism. Circ. Res. 35: 439-447, 1974.

Miller, T.B.: Cardiac performance of isolated perfused hearts from alloxan diabetic rats. Am. J. Physiol. 236: H808-H812, 1979.

Miller, T.B., Praderio, M., Wolleben, C. and Bullman, J.: A hypersensitivity of glycogen phosphorylase activation in hearts of diabetic rats. J. Biol. Chem. 256: 1748-1753, 1981.

Modrak, J.: Collagen metabolism in the myocardium from streptozotocin-diabetic rats. Diabetes 29: 547-550, 1980.

Moe, G.K. and Farah, A.E.: Digitalis and allied cardiac glycosides. In: The Pharmacological Basis of Therapeutics, 5th Ed., ed. by L.S. Goodman and A. Gilman, pp. 653-682, Collier, Macmillan, Toronto, 1975.

Mordes, J.P. and Rossini, A.A.: Animal models of diabetes. Am. J. Med. 70: 353-360, 1981.

Murthy, V.K. and Shipp, J.C.: Heart triglyceride synthesis in diabetes: selective increase in activity of enzymes of phosphatidate synthesis. J. Mol. Cell. Cardiol. 12: 299-309, 1980.

Neely, J.R., Bowman, R.H. and Morgan, H.E.: Effects of ventricular pressure development and palmitate on glucose transport. Am. J. Physiol. 216: 804-811, 1969.

Okita, G.T., Richardson, F. and Roth-Schechter, B.F.: Dissociation of the positive inotropic action of digitalis from inhibition of sodium potassium activated adenosine triphosphatase. J. Pharmacol. Exp. Ther. 185: 1-11, 1973.

Ohji, T. and Liu, M.: Effects of alloxan-diabetes on the sodium-potassium adenosine triphosphatase enzyme system in dog hearts. Biochem. Biophys. Res. Comm. 96: 799-804, 1980.

Opie, L.H., Mansford, K.R.L. and Owen, P.: Effects of increased heart work on glycolysis and adenine nucleotides in the perfused heart of normal and diabetic rats. Biochem. J. 124: 475-490, 1971.

Paulson, D.J. and Crass, M.F.: Myocardial triglycerol fatty acid composition in diabetes mellitus. Life Sci. 27: 2237-2243, 1980.

Paulson, D.J. and Light, K.E.: Elevation of serum and ventricular norepinephrine content in the diabetic rat. Res. Com. Chem. Path. Pharmacol. 33: 559-562, 1981.

Penpargkul, S., Fein, F., Sonnenblick, E.H. and Scheuer, J.: Depressed cardiac sarcoplasmic reticular function from diabetic rats. J. Mol. Cell. Cardiol. 13: 303-309, 1981.

Penpargkul, S., Schaible, T., Yipintsoi, T. and Scheuer, J.: The effect of diabetes in performance and metabolism of rat hearts. Circ. Res. 47: 911-921, 1980.

Pierce, G.N. and Dhalla, N.S.: Cardiac myofibrillar ATPase activity in diabetic rats, J. Mol. Cell. Cardiol. 13: 1063-1069, 1981.

Randle, P.J., Gardland, P.B., Hales, C.N., Newsholme, E.A., Denton, R.M. and Pogson, C.I.: Protein hormones. Interactions of metabolism and the physiological role of insulin. Recent Prog. Horm. Res. 22: 1-48, 1966.

Regan, T.J., Ettinger, P.O., Khan, M.I., Jesrani, M.U., Lyons, M.M., Oldewurtel, H.A. and Weber, M.: Altered myocardial function and metabolism in chronic diabetes mellitus without ischemia in dogs. Circ. Res. 35: 222-237, 1974.

Regan, T.J., Lyons, M.M., Ahmed, S.S., Levinson, G.E., Oldewurtel, H.A., Ahmed, M.R. and Haider, B.: Evidence for cardiomyopathy in familial diabetes mellitus. J. Clin. Invest. 60: 885-899, 1977.

Regan, T.J., Wu, C.F., Yeh, C.K., Oldewurtel, H.A. and Haider, B.: Myocardial composition and function in diabetes: the effect of chronic insulin use. Circ. Res. 49: 1268-1277, 1981.

Repke, K., Est, M. and Portius, H.J.: Uber die ursache de species-unterschiede in der digitalis empfindlichkeit. Biochem. Pharmacol. 14: 1785-1802, 1965.

Rerup, C.C.: Drugs producing diabetes through damage of the insulin secreting cells. Pharmacol. Rev. 22: 485-510, 1970.

Resh, M.D., Nemenoff, R.A. and Guidotti, G.: Insulin stimulation of (Na^+, K^+)-adenosine triphosphatase-dependent $^{86}Rb^+$ uptake in rat adipocytes. J. Biol. Chem. 255: 10938-10945, 1980.

Rhee, H.M., Dutta, S. and Marks, B.H.: Cardiac NaKATPase activity during positive inotropic and toxic action of ouabain. Eur. J. Pharmacol. 37: 141-153, 1976.

Robinson, J.D. and Flashner, M.S.: The $(Na^+ + K^+)$-activated ATPase: enzymatic and transport properties. Biochim. Biophys. Acta. 549: 145-176, 1979.

Rossini, A.A., Arcangeli, M.A. and Cahill, G.F., Jr.: Studies on alloxan toxicity on beta cell. Diabetes 24: 516-522, 1975.

Roth-Schechter, B.F., Okita, G.T., Thomas, R.E. and Richardson, F.F.: On the positive inotropic action of alkylating bromoacetates of strophanthidin and strophanthidol-(19^3H). J. Pharmacol. Exp. Ther. 171: 13-19, 1970.

Rubler, S., Sajadi, R.M., Araoye, M.A. and Holford, F.D.: Noninvasive estimation of myocardial performance in patients with diabetes. Diabetes 27: 127-134, 1978.

Ruoho, A. and Kyle, J.: Photoaffinity labeling of the ouabain-binding site on the Na^+, K^+-ATPase. Proc. Natl. Acad. Sci. 71: 2352-2356, 1974.

Savarese, J.J. and Berkowitz, B.A.: β adrenergic receptor decrease in diabetic rat hearts. Life Sci. 25: 2075-2078, 1979.

Schwartz, A., Grupp, I., Adams, R., Powell, T., Grupp, G. and Wallick, E.T.: Pharmacological and biochemical studies on the digitalis receptor. Abs. Third Inter. Conf. Na,K-ATPase 3: 20, 1981.

Sellers, B.M. and Ku, D.D.: Effects of ouabain on myocardial sodium pump activity and contractile force of STZ induced diabetic rats. Fed. Proc. 40: 663, 1981.

Sen, A.K. and Post, R.L.: Stoichiometry and localization of adenosine triphosphate-dependent sodium and potassium transport in the erythrocyte. J. Biol. Chem. 239: 345-352, 1964.

Senges, J., Brachmann, J., Pelzer, D., Hasslacher, C., Weihe, E. and Rubler, W.: Altered cardiac automaticity and conduction in experimental diabetes mellitus. J. Mol. Cell. Cardiol. 12: 1341-1351, 1980.

Shug, A.L., Shrago, E., Bittar, N., Folts, J.D. and Koke, J.R.: Acyl-CoA inhibition of adenine nucleotide translocation in ischemic myocardium. Am. J. Physiol. 228: 689-692, 1975.

Siperstein, M.D., Unger, R.H. and Madison, G.L.: Studies of muscle capillary basement membranes in normal subjects, diabetic, and pre-diabetic patients. J. Clin. Invest. 47: 1973-1999, 1968.

Skou, J.C.: The influence of some cations in adenosine triphosphatase from peripheral nerves. Biochim. Biophys. Acta. 23: 394-401, 1957.

Skou, J.C.: Further investigations in a Mg^{++} + Na^+ - activated adenosine triphosphatase, possibly related to the active, linked transport of Na^+ and K^+ across the nerve membrane. Biochim. Biophys. Acta. 42: 6-23, 1960.

Stephenson, R.D.: A modification of receptor theory. Br. J. Pharmacol. 11: 379-393, 1956.

Tada, M., Yamamoto, T. and Tonomura, Y.: Molecular mechanism of active calcium transport by sarcoplasmic reticulum. Physiol. Rev. 58: 1-79, 1978.

Tobin, T. and Brody, T.M.: Rates of dissociation of enzyme-ouabain complexes and $K0.5$ values in (Na^+ + K^+) adenosine triphosphatase from different species. Biochem. Pharmacol. 21: 1553-1560, 1972.

Tobin, T., Henderson, R. and Sen, A.K.: Species and tissue differences in the rate of dissociation of ouabain from (Na^+K^+)ATPase. Biochim. Biophys. Acta. 274: 551-555, 1972.

Vadlamudi, R. and McNeill, J.H.: Cardiac function in normal and diabetic rats. Proc. West. Pharmacol. Soc. 23: 29-31, 1980.

Vadlamudi, R. and McNeill, J.H.: Effect pf chronic streptozotocin induced diabetes in cardiac performance in rats. Proc. West. Pharmacol. Soc. 24: 73-77, 1981,a.

Vadlamudi, R.V.S.V. and McNeill, J.H.: Effect of short term experimental diabetes on cardiac performance in the rat. Proc. Can. Fed. Biol. Soc. 24: 193, 1981,b.

Vadlamudi, R.V.S.V. and McNeill, J.H.: Long term diabetes induced changes in cardiac function in the rat. The Pharmacologist. 23: 220, 1981,c.

Vadlamudi, R. and McNeill, J.H.: Isoproterenol induced changes in cyclic AMP, phosphorylase and inotropy in normal and diabetic rat hearts. Proc. Can. Fed. Biol. Soc. 25: 89, 1982.

Vadlamudi, R.V.S.V., Rodgers, R.L. and McNeill, J.H.: The effect of chronic alloxan - and streptozotocin - induced diabetes on isolated rat heart performance. Can. J. Physiol. Pharmacol. 60: in press, 1982.

Vaisrub, S.: Diabetes and the heart: the autonomic connection. In Diabetes and the Heart, ed. by S. Zoneraich, pp. 161-174, Chas. C. Thomas, Springfield, Ill., 1978.

Wellsmith, N.V. and Lindenmeyer, G.E.: Two receptor forms for ouabain in sarcolemma-enriched preparations from canine ventricle. Circ. Res. 47: 710-720, 1980.

West, K.M.: Current concepts, natural history, classification, and definitions. In Epidemiology of Diabetes and its Vascular Lesions, pp. 19-39, Elsevier, New York, 1978a.

West, K.M.: Factors associated with development of diabetes. In Epidemiology of Diabetes and its Vascular Lesions, pp. 191-285, Elsevier, New York, 1978,b.

Yamamoto, S., Akera, T., Kim, D. and Brody, T.M.: Tissue concentration of Na^+K^+ adenosine triphosphatese and the positive inotropic action of ouabain in guinea pig heart. J. Pharmacol. Exp. Ther. 217: 701-707, 1981.