MOLECULAR ABNORMALITIES IN FAILING HEARTS OF GENETICALLY MYOPATHIC HAMSTERS

A Thesis

Presented to the

University of Manitoba

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

by

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June, 1971

ABSTRACT

The ability of sarcoplasmic reticulum and mitochondria, isolated from the control and failing hearts of genetically myopathic hamsters (BIO 14.6 strain) with a moderate degree of failure, to accumulate calcium was examined. The rate and extent of energy-linked calcium binding (in the absence of oxalate) by the sarcoplasmic reticulum of the failing heart were markedly depressed. The calcium uptake (in the presence of 5 mM oxalate) by the sarcoplasmic reticulum of the failing heart was similar to that of the control heart. On the other hand, both the rate and extent of energy-linked calcium binding (in the absence of Pi and succinate) and calcium uptake (in the presence of 4 mM Pi and 5 mM succinate) by mitochondria were greatly reduced in the failing heart in comparison to the control. No difference in the total ATPase activities (Ca^{++} - Mg^{++} stimulated) of sarcoplasmic reticulum or mitochondria was observed between the control and failing hearts. The kinetic analysis of the results on calcium transport by the mitochondria of the failing hearts revealed no changes in the affinity of sites for calcium while V_{max} (maximum velocity of reaction) was markedly depressed. These results indicate an abnormality of subcellular membranes of the failing heart to bind calcium and support the growing conviction concerning the defective "calcium pump" as a molecular abnormality associated with a moderate degree of heart failure.

The kinetics of calcium transport by the heavy microsomal fraction of the myopathic hearts at an advanced degree of failure was also investigated. The calcium uptake in the presence of 5 mM Pi or 5 mM oxalate was markedly depressed in these preparations. The affinity for Mg^{++} of the low affinity binding

sites of heavy microsomal membranes was unaltered in myopathic hearts. The affinity for ATP (K_m) of the low affinity binding sites of heavy microsomes from myopathic hearts was same as that of control whereas the K_m value of the high affinity binding sites was increased in failing hearts. The affinity constants for calcium were the same whereas V_{max} for the heavy microsomes of myopathic hearts was lower than control. This defect in calcium uptake by the heavy microsomes in late stages of heart failure may represent an irreversible damage to these membranes.

In another series of experiments, the activities of $Na^+ - K^+$ ATPase of the control and failing hearts of myopathic hamsters with a moderate degree of failure were determined. The activity of this enzyme from failing hearts was observed to be higher than the control under various experimental conditions such as changes in the concentrations of different ions and ATP in the incubation media. The K_m value of $Na^+ - K^+$ ATPase from failing hearts was the same as that of the control while V_{max} was markedly increased. These enzymes isolated from the control and failing hearts exhibited typical allosteric characteristics and showed similar sensitivities to inhibition by ouabain and calcium. It is suggested that alteration in the activity of $Na^+ - K^+$ ATPase, which is considered to be involved in the transport of Na^+ and K^+ across heart sarcolemma, may represent one of the compensatory mechanisms in myopathic hamsters with a moderate degree of heart failure.

The activities of adenyl cyclase, another membrane bound enzyme important for the regulation of myocardial metabolism, were also measured in failing hearts in the absence or presence of its well known activators, norepin-

ephrine and NaF. No appreciable change in enzyme activities in homogenates and washed cell particles was observed in hearts with a moderate degree of failure. However, at the advanced stages of heart failure, the responses of this enzyme to fluoride were markedly reduced and those to norepinephrine were not apparent. The basal adenyl cyclase activities in the failing hearts were not different from the control. These results indicate that alterations in adenyl cyclase activities in the presence of NaF and norepinephrine are secondary to heart failure in myopathic hamsters but strengthen our belief concerning a defect in cardiac membranes in heart failure.

ACKNOWLEDGEMENTS

It gives me a great pleasure to express my very sincere thanks to Dr. Naranjan S. Dhalla, my supervisor and friend, for his most encouraging and able guidance and valuable advice on many occasions, including the preparation of this manuscript. The unique opportunity provided by Dr. Dhalla has been a major factor in developing my understanding and interests in cardiac and skeletal muscle physiology. Special thanks are also due to Dr. K.R. Hughes, Graduate Program Director of this department, for kind interest in my training. I am grateful to Professor A. Naimark, Chairman of the Department, for his encouragement during the course of my studies in this University.

I wish to offer my appreciation to my colleagues and friends, especially Dennis (Ben) McNamara, Ramji Khandelwal, Charles Tomlinson, Irwin (Joe) Borowski, Kenneth Varley, V. Balasubramanian, Margaret Fedelesova, Daniel Houle, John Yates, Margaret Davies and Ivan Toffler for their scientific interaction and co-operation. My thanks are also due to Dr. E.T. Pritchard, Dr. C. Dowse, and Dr. I.R. Hamilton for allowing me the use of certain equipments. Expert secretarial help of Miss Chris Chubaty is greatfully acknowledged.

The study was completed while the author was a demonstrator and teaching fellow of the Department of Physiology. The cost of this research was defrayed from the funds provided by the Medical Research Council of Canada and the Manitoba Heart Foundation to Dr. N.S. Dhalla.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION AND STATEMENT OF THE PROBLEM

In various laboratories extensive attempts are being made to find a specific biochemical abnormality which may be considered responsible for heart failure. Some investigators have demonstrated a defect in energy production (1 - 5), while others have claimed an impairment of the mechanisms for energy storage and utilization (6 - 10). Reports have also appeared in the literature showing normal energy metabolism in the failing heart (11 - 16). These studies provide evidence for the traditional concept that heart failure is a condition of multiple etiology. The data published so far essentially support the hypothesis of Olson (17) that there exists at least two molecular classes of heart failure, i.e. those in which the defect lies in the generation of ATP and those in which the defect lies in in the utilization of ATP. It has been suggested that these "molecular forms" of heart failure correspond to high output failure and low output failure respectively.

The object of the present research is to examine a new concept concerning the molecular basis for the pathogenesis of heart failure. This concept is centered around an abnormality in cardiac membranes and is tested by employing a special strain of genetically myopathic hamsters (BIO 14.6) as an experimental model. Due to certain difficulties in procuring these hamsters we have chosen to use only two groups of these animals - one with a moderate degree of heart failure (210 - 230 day old animals) and the other with an advanced degree of heart failure (260 - 275 day old animals). These animals offer a unique opportunity to study biochemical events in a naturally occuring form of myocardial failure (18).

The main feature of this study deals with the identification of a defect

in the ability of mitochondria and heavy microsomal fractions of the failing heart to transport calcium. The "calcium pump" in these membranes is characterized in terms of its kinetic parameters under different experimental conditions. Fractions containing $Na^+ - K^+$ ATPase activity are isolated from the control and myopathic hearts. The activities of these fractions, which are presumably derived from heart sarcolemma, are measured under a variety of experimental conditions. The activity of adenyl cyclase, another membrane bound enzyme, is tested in the absence or presence of various concentrations of its well known activators, such as NaF and norepinephrine. These experiments are performed by employing both whole heart homogenates and washed cell particles in order to obtain further information concerning the integrity of cardiac membranes of the failing heart. The alterations in the activities of both $Na^+ - K^+$ ATPase and adenyl cyclase, in addition to changes in the "calcium pump" mechanisms in mitochondria and heavy microsomes, are hoped to form the molecular basis of membrane defect in heart failure.

REVIEW OF LITERATURE

1. Current State of Knowledge Concerning Molecular Abnormalities in Failing Heart

Until recently, development of our insight into the mechanisms of heart failure has been based on the traditional vascular-oriented approach concerning the broad spectrum of largely non-vascular pathogenic elements involved. Studies on the complex issues of fluid retention as well as mechanisms affecting vascular resistance and volume at extracardiac sites (19, 20) have not been designed to provide information regarding the molecular abnormality leading to failure of heart function. Recent heart transplant experiments have provided dramatic illustration that the defect in heart failure is in the myocardium itself while all other well known symptoms are secondary in nature. It is therefore considered of vital importance that efforts should be directed toward the study of contractile process both in normal and failing hearts.

Heart failure due to valvular insufficiencies and stenoses has been recognized on the basis of chamber enlargement associated with an increase in diastolic pressure (21) and a general concept of "overloading of the heart" has been commonly used in defining the clinical condition of myocardial failure. Sarnoff and Berglund (22) have suggested that insufficiency of the atrioventricular valve which may occur at high ventricular volume may be a primary cause of the descending limb of the ventricular function curve. Aortic constriction was reported to produce an increase in left ventricular and diastolic pressure indicating that the left ventricle can make use of the Starling effect in adjusting to outflow obstruction (23). Acute pulmonary artery constriction involves increase in peak ventricular

pressure without concomitant increase in end diastolic pressure, under certain limits (24). Such classical studies have helped to develop techniques for inducing experimental heart failure and to establish functional criteria for recognizing its presence. Unfortunately they do not reveal the underlying mechanisms.

The work reviewed by Bing (25) has supported the view that in ischemic heart disease the defect in energy production leads to heart failure. However, it is not clear whether such an abnormality plays a primary role in congestive heart failure. Numerous investigators have examined oxygen, glucose, lactate and free fatty acid utilization in the failing heart and have concluded that utilization of these substrates is not diminished (13, 26). The work of Schwartz and Lee (2) and of Wollenberger et al (1) suggests a mitochondrial abnormality in the failing heart whereas no such defect was observed by Plaut and Gertler (14), Olson (27) and Chidsey et al (12). A reduction in the content of high energy phosphate compounds (ATP and creatine phosphate) was reported to be associated with the failure of heart by some investigators (28, 29) and was denied by others (12, 30). The onset of heart failure due to substrate-lack was found to be associated with an abnormality in the process of energy generation (31, 32).

The possible abnormality in the process of energy utilization in the failing heart seems more attractive but, unfortunately, lacks direct evidence. A defect in energy utilization in heart failure was considered due to a dissociation of an actomyosin complex (33), due to a derangement of the quaternary structure of myosin (34), and due to a reduction in ATPase activity of myosin (8 - 10), but these findings have been challenged by the work of Davis et al (35, 36) and Mueller et al (37). It should be recognized that the utilization of stored energy by the

heart muscle is a complex process which also involves the excitation-contraction coupling mechanism, in addition to a chemical reaction between actin and myosin. The energy is also utilized in various other synthetic processes as well as for the maintenance of ionic gradients across the cell membrane.

In myocardial failure, the contractile elements of the ventricle exhibit a diminished ability to develop tension, a decreased rate of shortening and a diminished extent of shortening (38). Slippage of muscle fibres (39) and a reduction in the extent of overlap of actin and myosin filaments in the sarcomere (40) are considered to form the ultrastructural basis of heart failure. In an experimental model using isolated rat heart perfused with substrate-free medium, Dhalla et al (32) have shown a marked decrease in myocardial contractility without any changes in the ultrastructure of the cardiac muscle. Therefore, further studies in this direction are highly warranted for a meaningful conclusion.

Braunwald and associates (41 - 48), have reported that a defect in endogenous norepinephrine stores may account for the heart failure. It was shown that norepinephrine concentration in the arterial blood during exercise or daily urinary excretion of norepinephrine at rest from patients with heart failure was much larger than in normal subjects. Furthermore, the blockade of adrenergic receptors with propranolol or blockade of adrenergic neurons with guanethidine impaired cardiac performance in patients with heart failure. In heart failure, the myocardium becomes profoundly depleted of norepinephrine stores (46). The ability to release norepinephrine and the response to sympathetic nerve stimulation are reduced (44) which in turn support the concept that the failing heart is unable to modulate contractile force through neurogenic pathways (43). These results

also suggest that the hyperactivity of the adrenergic nervous system due to the depletion of catecholamines from the myocardial stores may play an important role in the maintenance of heart performance in the initial stages but later on may further complicate the problem of heart failure. Recently, we have shown a dramatic shift of the endogenous norepinephrine in the subcellular fractions of the failing heart, however, we have failed to reveal any relationship between changes in catecholamine stores and cardiac contractile force in the isolated hearts perfused with substrate-free medium (49).

The involvement of a defect in the excitation-contraction coupling mechanism (50, 51) in heart failure has been suspected by various investigators (52 - 55). The current concept of excitation-contraction coupling implies that the release of calcium from a superficial membrane site in the cell in response to depolarizing impulse, results in myocardial contraction by activating the contractile apparatus. In the heart, sarcoplasmic reticulum, mainly, and mitochondria to some extent are considered to bring about relaxation due to their abilities to sequester calcium from sarcoplasm by energy dependent mechanisms (56 - 63). Thus, the ability of subcellular structures to regulate intracellular calcium constitutes an important factor for determining the contractile state of myocardium.

Numerous investigators have attempted to show an abnormality of sarcotubular vesicles to accumulate calcium in heart failure induced by different procedures. For example, Gertz et al (64) have reported that the ability of sarcoplasmic reticulum to accumulate calcium was markedly impaired in spontaneously failing dog heart-lung preparation. A decrease in both the rate and extent of calcium accumulation by the sarcoplasmic reticulum of the ischemic dog

heart muscle was also demonstrated (65). Harigaya and Schwartz (66) have shown a reduced rate of calcium binding by the sarcoplasmic reticulum isolated from the failing human heart. The work of Suko et al (67) using sarcoplasmic reticulum obtained from the right ventricle of calves with right ventricular failure due to chronic pulmonary hypertension, also reveals a defect in calcium pump mechanism. Furthermore, a lesion in the energy-linked calcium transport across the mitochondria and sarcoplasmic membranes was observed in the failing rat heart on perfusion with substrate-free medium (56, 68). None of these investigators have studied the ability of subcellular fractions from the failing heart to release calcium. At any rate, these observations indicate membrane abnormality in heart failure.

Some of the observations made by various investigators concerning biochemical changes in the failing heart can be interpreted to suggest an abnormality of cardiac sarcolemma in heart failure. For example, the basal activity of myocardial adenyl cyclase as well as its responses to fluoride were decreased in congestive heart failure produced by constriction of the ascending aorta in guinea pigs (69). On the other hand, other investigators (70 - 72) failed to demonstrate any change in myocardial adenyl cyclase activity in the absence or presence of norepinephrine and fluoride in chronic heart failure produced by occluding the pulmonary artery in cats; however, adenyl cyclase activation by glucagon was lost in this preparation. Although a recent report indicates no changes in adenyl cyclase activity in the failing guinea pig heart (73), the work from this laboratory on heart failure induced by substrate-lack in isolated perfused rat hearts reveals that changes in adenyl cyclase activity in failing heart are dependent upon the degree of heart failure (74). It should be mentioned that adenyl cyclase is a

membrane bound enzyme and is considered to be mainly localized in the cell membrane (75 - 77). Since adenyl cyclase catalyzes the formation of cyclic AMP, which is an important regulator of cell metabolism (75, 78, 79), any alteration in its activity may constitute one of the crucial factors leading to derangements in myocardial metabolism.

Although no report concerning the integrity of cardiac sarcolemma in failing heart is available, some changes in the activity of $Na^+ - K^+$ ATPase have been reported in heart failure. Recently, Allan and Schwartz (80) have indicated that the activity of $Na^+ - K^+$ ATPase from the failing digitilized human heart was lower in comparison to the normal. Unfortunately, it is difficult to comment on such an observation since digitalis itself is known to inhibit $Na^+ - K^+$ ATPase. Another group of investigators (81) has shown that in cardiac failure produced by aortic constriction or renal artery stenosis, $Na^+ - K^+$ ATPase activity was decreased. Such studies support the contention concerning the molecular abnormality in cardiac sarcolemma in heart failure. It should be noted that $Na^+ - K^+$ ATPase is considered to be involved in the transport of Na^+ and K^+ across the cell membrane (82, 83). Recent reports (84 - 86) have indicated its presence in the heart sarcolemma.

2. General Background of Work on Myopathic Hamsters as an Experimental Model for Heart Failure

A special strain of Syrian hamsters (BIO 14.6), which develop a hereditary cardiomyopathy, has been regarded as an excellent model for studying the pathogenesis of heart failure (87 - 91). The cardiac function in these animals has been shown to be markedly depressed (92 - 94). These animals are unique in the sense that their death occurs due to congestive heart failure and

different age groups of these animals show a varying degree of heart failure. The onset of heart failure is considered to occur at the age of 5 to 6 months, whereas 7 to 8 month old animals usually exhibit a moderate degree of heart failure. These animals normally die at the age of 9 to 10 months and thus 260 to 275 day old animals are considered to be at an advanced degree of heart failure.

Focal myocardial degeneration occurs spontaneously in all myopathic hamsters and the underlying defect is transmitted by an autosomal recessive gene (90). Myocardial hypertrophy, fluid retention, pulmonary edema, lung and liver congestion, and cardiocirculatory insufficiency are the main features of these animals (91, 95). Myopathic hamsters yielded hemodynamic evidence of depressed ventricular function: bradycardia, elevation of both right and left ventricular filling pressures, decrease in systemic blood pressure, and decrease in ventricular dp/dt (96). Serum phosphocreatine kinase level was also found to be higher in myopathic hamsters (97).

Some investigators (98 - 101) have reported an abnormality of oxidative phosphorylation in the hearts of these myopathic hamsters while others have failed to observe such a lesion (102). Lochner et al (99) have shown a small decrease in the level of creatine phosphate; however, it is difficult to interpret changes in the high energy phosphate stores in the myopathic hearts because the value of creatine phosphate for the control heart reported by these workers is far below the accepted level for the normal myocardium. In another report, Lochner et al (94) have indicated a depression of creatine phosphate, ATP and AMP levels without any changes in the levels of ADP in the isolated perfused hearts of myopathic hamsters. Recently Fedelesova and Dhalla (103) have claimed that the changes

in the high energy phosphate stores in the hearts of myopathic animals are similar to those in the asphyxiated hearts and on the basis of dramatic alteration in myocardial metabolism these workers have suggested a defect in the process of energy generation.

Mitochondria isolated from hearts of cardiomyopathic animals accumulated less calcium in comparison to the control and the magnitude of these changes in calcium uptake were shown to be related to the severity of heart failure (100, 101). Another group of investigators (104) has shown that the ATP dependent Ca^{++} oxalate pumping of the sarcoplasmic reticulum was reduced in the hearts of myopathic hamsters. The quantity of sarcoplasmic reticulum in the tissue as judged by the Ca^{++} oxalate capacity of homogenate was also decreased in the failing hearts. Myopathic heart reticular fraction also showed a decrease in rate and maximal capacity of calcium binding (105). The above mentioned reports appeared in the literature when the present work was being carried out in this laboratory. A part of the work described here has already been published (106). These studies indicate a defect in calcium transport by the subcellular fractions of the hearts of myopathic hamsters. A preliminary report concerning molecular abnormalities in myopathic hearts has been published elsewhere (107).

METHODS

1. Studies on Calcium Transport

a) Isolation of Subcellular Fractions

Control and myopathic hamsters (BIO 14.6) were decapitated, hearts quickly removed and subcellular fractions isolated according to the following methods:

Method "A". After thoroughly washing the hearts with 0.25 M sucrose containing 1 mM EDTA, pH 7.0, the tissue was homogenized in 10 volumes of medium (10 mM sodium bicarbonate, 5 mM sodium azide and 15 mM Tris-HCl, pH 6.8) in a VirTis homogenizer for 20 seconds (10 seconds x 2). The homogenate was filtered through 4 layers of gauze and centrifuged at 1000 x g for 10 minutes and then at 10,000 x g for 20 minutes to remove cell debris, nuclei, myofibrils and mitochondria. The residue was discarded and the supernatant was spun at 40,000 x g for 45 minutes. The sediment thus obtained was washed thoroughly, suspended in 0.6 M KCl containing 20 mM Tris-HCl, pH 6.8 and centrifuged at 40,000 x g for 45 minutes. This procedure was repeated and the final pellet (sarcoplasmic reticulum, heavy microsomes or sarcotubular vesicles) was suspended in 50 mM KCl, 20 mM Tris-HCl, pH 6.8 at a protein concentration of 3 to 5 mg/ml. This method of isolation of the heavy microsomes is essentially similar to that described by Harigaya and Schwartz (66) in which azide was employed in the homogenizing medium. The addition of azide in this homogenizing medium yielded a very active preparation of cardiac sarcoplasmic reticulum in terms of calcium pump with minimal contribution of mitochondrial fragments.

Method "B". For isolating mitochondria the hearts were homogenized

with 10 volumes of medium (0.18 M KCl, 10 mM EDTA, 0.5% albumin (fatty acid free), pH 7.4) in a VirTis homogenizer for 20 seconds (10 seconds x 2). The homogenate, after filtering through 4 layers of gauze, was spun at 1000 x g for 20 minutes to remove cell debris, nuclei, and myofibrils. The supernatant was spun at 10,000 x g for 20 minutes. The sediment thus obtained was gently suspended in the homogenizing medium, centrifuged at 1000 x g for 10 minutes and the residue discarded. The supernatant was further centrifuged at 8,000 x g for 10 minutes. This washing procedure was repeated and the final pellet (mitochondria) was suspended in 50 mM KCl, 20 mM Tris-HCl, pH 6.8 at a protein concentration of 3 to 6 mg/ml. This procedure for isolating mitochondria is similar to that described by Sordahl and Schwartz (108).

Method "C". By this method both mitochondria and heavy microsomes were isolated from the same tissue. The heart was homogenized with 10 volumes of medium (0.25 M sucrose, 1 mM EDTA, pH 7.0) in a VirTis homogenizer for 40 seconds (20 seconds x 2). The homogenate was filtered through gauze, centrifuged at 1,000 x g for 20 minutes to remove cell debris and the supernatant was centrifuged at 10,000 x g for 20 minutes to obtain mitochondrial sediment. This sediment was washed and suspended in the homogenizing medium, spun at 1,000 x g for 10 minutes, the residue discarded and the supernatant further centrifuged at 8,000 x g for 10 minutes to obtain mitochondrial fraction. The post 10,000 x g supernatant was centrifuged at 40,000 x g for 45 minutes, the sediment washed, resuspended in 0.6 M KCl containing 20 mM Tris-HCl, pH 6.8 - 7.0, and centrifuged at 40,000 x g for 45 minutes to separate heavy microsomes. Both mitochondrial and reticular fractions obtained by this method were suspended in

0.25 M sucrose, 10 mM Tris-HCl, pH 7.0 at a protein concentration of 2 to 5 mg/ml.

Method "D". In experiments where liver was used, the tissue was cut into small pieces by a pair of scissors, washed thoroughly in a medium containing 0.25 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 7.0 and homogenized by hand with 10 volumes of the same medium in a glass teflon homogenizer (6 to 8 strokes). The homogenate after filtering through 2 layers of gauze was spun at 1000 x g for 15 minutes, the residue discarded and the supernatant centrifuged at 8,500 x g for 15 minutes. The sediment thus obtained was suspended in the homogenizing solution and again spun at 1000 x g for 10 minutes, the residue discarded and the supernatant centrifuged at 8,500 x g for 10 minutes. This procedure was repeated and the mitochondrial fraction thus obtained was suspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.0 at a protein concentration of 5 to 10 mg/ml.

b) Measurement of Calcium Uptake by Heavy Microsomes

The calcium uptake by the heavy microsomes was measured by incubating these particles (membrane protein conc. 0.02 to 0.05 mg/ml) in medium containing 100 mM KCl, 10 mM $MgCl_2$, 5 mM potassium oxalate, 20 mM Tris-HCl, pH 6.8, 4 mM ATP and 0.1 mM $Ca^{45}Cl_2$ in a total volume of 2 ml. The reaction was started by the addition of membrane protein and was stopped by millipore filtration (0.45μ) at various times of incubation at 37°C. The protein-free filtrate (0.1 ml) was mixed with 15 ml of Bray's solution (109) and the radioactivity was estimated in a Packard liquid scintillation spectrometer (Series 3375). The millipore filters effectively remove the protein and stop the reaction. This was checked by the estimation of protein from the filtrates. Not more than 0.5 ml of incubation

mixture was filtered at any one time and the filtration was completed within 2 - 3 seconds. The amount of membrane protein in the incubation mixture to be filtered at any one time did not exceed more than 300 μg. The use of plastic disposable syringes also aided in the rapidity and reproducibility of this technique.

c) Measurement of Calcium Binding by Heavy Microsomes

The calcium binding by heavy microsomes was carried out at 25°C in the same medium as described for calcium uptake except that potassium oxalate was omitted and the protein concentration in the reaction medium was 0.25 to 0.35 mg/ml. Other details are the same as described for the calcium uptake by heavy microsomes.

d) Calcium Uptake by Mitochondria

The incubation medium employed for estimating mitochondrial calcium uptake contained 100 mM KCl, 20 mM Tris-HCl, pH 6.8, 4 mM Pi, 5 mM sodium succinate, 4 mM ATP, 10 mM $MgCl_2$ and 0.1 mM $Ca^{45}Cl_2$ (mitochondrial protein concentration 0.2 to 0.3 mg/ml) at 37°C. All other details are similar to those described for calcium uptake by heart heavy microsomes.

e) Calcium Binding by Mitochondria

Calcium binding by mitochondria was carried out at 25°C in the same medium as for mitochondrial calcium uptake except Pi and sodium succinate were omitted from the reaction medium and the protein concentration was 0.3 to 0.5 mg/ml. Other details are as described for calcium uptake by heavy microsomes.

f) ATP-Independent Calcium Binding

ATP-independent calcium binding by the subcellular fractions was

determined by incubating these particles for 5 minutes at 25°C in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, 0.1 mM $Ca^{45}Cl_2$ and membrane protein (0.2 to 0.3 mg/ml). The reaction was stopped by millipore filtration. The other details are similar to those described above.

g) Calcium Content of the Subcellular Particles

The total calcium content of the subcellular particles was measured by Zeiss atomic absorption spectrophotometer after extracting with 0.5 N HCl according to the method described by Reynafarje and Lehninger (110). $LaCl_3$ (1%) was added to eliminate the interference by other ions during the determination of calcium by atomic absorption spectrophotometry.

h) Measurement of Ca^{++} - Mg^{++} ATPase Activity

The ATPase activity was determined by incubating the subcellular particles in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 4 mM ATP, 5 mM K-oxalate, 0.1 mM $CaCl_2$, 20 mM Tris-HCl, pH 6.8 at 37°C. The protein concentraion was 0.02 to 0.05 mg/ml. The Pi released due to the hydrolysis of ATP was determined in the protein-free filtrate at 1, 5 and 10 minutes of incubation by the method of Fiske and SubbaRow (111).

i) Measurement of Marker Enzyme Activities

Glucose-6-phosphatase, 5'-nucleotidase and cytochrome c oxidase activities of the subcellular fractions were determined according to the methods described elsewhere (112 - 114). The glucose-6-phosphatase and 5'-nucleotidase were used as markers for heavy microsomes while cytochrome c oxidase was used as a mitochondrial marker.

The essential details of these methods for calcium uptake and binding

by the subcellular particles and for the ATPase activity are also described elsewhere (56, 106, 115).

The subcellular fractions from the hearts of control and myopathic hamsters were prepared simultaneously and were used within 2 hours of their isolation. A uniform time between preparation of the subcellular fractions and measurement of calcium transport was kept in all experiments. The isolation of the subcellular particles was carried out at 0 - 4°C. The Sorvall RC-2B refrigerated centrifuge was employed for the isolation of the subcellular fractions. Any change in the experimental condition is described under the tables and figures.

2. Studies on $Na^+ - K^+$ ATPase

a) Isolation of $Na^+ - K^+$ Stimulated Mg^+ Dependent Ouabain Sensitive ATPase from Hamster Heart

Method "A". After decapitating the animals, the hearts were quickly placed in ice cold medium (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.2 to 7.4), washed thoroughly to remove blood, and homogenized with 15 volumes of the above medium for 40 seconds (20 seconds x 2) in a VirTis homogenizer in a cold room (0 - 4°C). The homogenate was filtered through several layers of gauze, centrifuged at 10,000 x g for 15 minutes, the residue homogenized (original volume) for 20 seconds with the above medium containing 0.1% deoxycholate, allowed to stand for 20 minutes, and spun at 10,000 x g for 15 minutes in a Sorvall RC-2B refrigerated centrifuge. The sediment was discarded and the clear supernatant, after filtration, centrifuged at 70,000 x g for 1 hour in an International Centrifuge, Model B-60 (Rotor #A-211). The residue was suspended in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.2 to 7.4 containing 0.05%

deoxycholate with the aid of a glass-teflon homogenizer, allowed to stand at 0 - 4°C for 15 to 20 minutes and then centrifuged at 10,000 x g for 10 minutes. The residue was discarded and the supernatant, after filtration, was centrifuged at 70,000 x g for 1 hour. The residue thus obtained was thoroughly washed and suspended in 1 mM Tris-EDTA solution (pH 7.0) and centifuged at 70,000 x g for 1 hour. This process was repeated twice, the sediment suspended in 1 mM Tris-EDTA, immediately assayed for enzyme activity, and then frozen at -20°C. The enzyme activity was stable for about 3 to 4 weeks on storage. This preparation (usually within one week) was further treated as follows: the suspension was stirred slowly in an ice bath with 2 M sodium iodide, 5 to 10 mM $MgCl_2$, 15 mM EDTA, 100 mM Tris-HCl, pH 7 to 7.2 for 45 minutes, diluted 2.5 times with 1 mM Tris EDTA, and centrifuged at 38,000 x g for 45 minutes. The final residue was obtained by a repeated cycle of washing, resuspending, and centrifuging in 1 mM Tris-EDTA and was assayed immediately for $Na^+ - K^+$ stimulated ATPase activity. The suspension was then divided into separate vials, quickly frozen and stored at -20°C. The enzyme activity was stable for at least 2 to 3 weeks. The enzyme preparation was used, however, within one week for the experiments to be reported here. The principal of this procedure is based on the method described by Matsui and Schwartz (116).

Method "B". The hearts were dissected quickly after decapitating the animals, and washed thoroughly to remove blood with 0.25 M sucrose, 10 mM Tris-HCl, pH 7.2 - 7.4, 1 mM EDTA. The tissue was homogenized with 15 volumes in 10 mM $NaHCO_3$, 5 mM sodium azide, 10 - 15 mM Tris-HCl, pH 6.8 for 20 seconds (10 seconds x 2) in a VirTis homogenizer at 0 - 4°C and the homogenate

after filtration through gauze, was centrifuged at 10,000 x g for 15 minutes. The residue was rehomogenized with 10 volumes of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.2 - 7.4, 1 mM EDTA containing 1% deoxycholate and the same procedure as described under Method "A" was followed.

b) Assay for Mg^{++} Dependent ATPase

The fractions were incubated at 37°C in a total volume of 1 ml containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 4 mM Tris-ATP for 10 minutes. The reaction was started by the addition of ATP and stopped by addition of 1 ml of cold 12% trichloracetic acid (TCA) and the inorganic phosphate (Pi) released was estimated from the clear supernatant obtained after centrifugation.

c) Assay for Na^{+} - K^{+} Stimulated Mg^{++} Dependent ATPase

The fractions were incubated in the medium containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 4 mM Tris-ATP, 100 mM NaCl, 20 mM KCl for 10 minutes at 37°C. The reaction was started by ATP and stopped by addition of 1 ml of cold 12% TCA, and released Pi was estimated from the clear supernatants. This represents the total ATPase activity. When the Mg^{++} dependent ATPase activity was subtracted from the total ATPase activity, the difference was taken as Na^{+} - K^{+} stimulated ATPase activity.

The Na^{+} - K^{+} stimulated Mg^{++} dependent ATPase was highly ouabain sensitive and 0.2 mM ouabain was able to inhibit the stimulation due to addition of 100 mM Na^{+} and 20 mM K^{+} completely. This concentration of ouabain had no effect on the Mg^{++} dependent ATPase activity. Therefore, for routine estimation of the Na^{+} - K^{+} stimulated ATPase activity, the fractions were incubated in total volume of 1 ml containing 50 mM Tris-HCl, pH 7.4 - 7.6, 1 mM

EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl in the presence or absence of ouabain (0.2 mM). After 5 minutes of preincubation at 37°C, the reaction was started by addition of ATP (4 mM final concentration) and the fractions were further incubated for 10 minutes. The reaction was stopped by the addition of 1 ml of 12% cold trichloroacetic acid and the Pi in the clear supernatants was estimated. The difference of the activities in the absence and presence of ouabain referred to $Na^+ - K^+$ stimulated Mg^{++} dependent ouabain sensitive ATP-ase ($Na^+ - K^+$ ATPase) while the activity in the presence of ouabain is referred to as Mg^{++} dependent ATPase (Basal ATPase). Any change in the experimental conditions is described under the tables and figures.

d) Estimation of Inorganic Phosphate

This was carried out by the method of Taussky and Shorr (117). Usually 0.5 ml or 1.0 ml of the clear supernatant was employed for the estimation of phosphate. Each time the standard curve for Pi was constructed to ensure the reliability of phosphate values. Occasionally, the phosphate values were also checked by the method of Fishe-SubbaRow (111). Protein concentration of the incubation assay was always kept within the limits of the linearity of the assay system. The reaction was linear for time of incubation used in the present investigation and not more than 15% of the added ATP was hydrolyzed under these conditions. This eliminated the necessity for the use of ATP-regenerating system during the assay of the ATPase activity. It should be mentioned that the use of ATP-regenerating system yielded comparable results as judged from some preliminary experiments. Protein concentration was estimated by the method of Lowry et al (118).

3. Studies on Adenyl Cyclase

a) Isolation of Particulate Fractions for the Adenyl Cyclase Activity

Method "A" (Isolation of Washed Cell Particles). The hearts were homogenized in 10 - 15 volumes of 50 mM Tris-HCl, pH 7.6 for 20 seconds (10 seconds x 2) in a VirTis homogenizer at 0 - 4°C in the cold room. The homogenate was filtered through several layers of gauze and centrifuged at 2200 x g for 15 minutes. The residue was thoroughly washed and suspended in the above buffer and centrifuged at 2200 x g for 15 minutes. This process was repeated once more. The residue thus obtained was suspended in 50 mM Tris-HCl, pH 7.6 and used immediately for the estimation of enzyme activity. This method is essentially similar to that described by Drummond and Duncan (119).

Method "B" (Isolation of Washed Cell Particles and Heavy Microsomes). The hearts were homogenized in 10 - 15 volumes of 250 mM sucrose - 10 mM Tris-HCl, pH 7.6 for 20 seconds (10 seconds x 2) in a VirTis homogenizer at 0 - 4°C. The remaining procedure for the isolation of washed cell particles is similar to that described above in Method "A" except for the use of sucrose-Tris buffer instead of Tris buffer. The original 2200 x g supernatant was centrifuged at 10,000 x g for 15 minutes to remove the mitochondrial fraction. The post 10,000 x g supernatant was further centrifuged at 40,000 x g for 45 minutes to separate heavy microsomes. This residue was suspended in 0.6 M KCl - 20 mM Tris-HCl, pH 7.0, allowed to stand for 30 minutes at 0°C and centrifuged at 40,000 x g for 45 minutes. The supernatant was discarded and the residue (heavy microsomes or sarcoplasmic reticulum) was suspended gently with the aid of a glass teflon homogenizer in 0.25 M sucrose - 10 mM Tris-HCl, pH 7.6 and used immediately for the enzyme

assay.

The particulate fractions (washed cell particles and heavy microsomes) as well as the original homogenate were assayed for the adenyl cyclase activity within 15 minutes of their isolation. Control and myopathic hearts were processed on the same day and a uniform time was kept between isolation of the fractions and assay of the enzyme activities.

b) Estimation of Adenyl Cyclase Activity

The adenyl cyclase activity was assayed at 37°C in a total volume of 0.1 ml containing 25 - 30 mM Tris-HCl, pH 7.4 - 7.6, 2 mM $MgCl_2$, 2 mM ATP-8-C^{14}, 8 mM theophylline or caffeine, 20 mM phosphoenol pyruvate, about 10 µg of pyruvate kinase, 5 - 10 mM KCl and 0.5 mg albumin/ml. The reaction was started by the addition of either fractions or ATP, and stopped by boiling the tubes for 4 minutes under conditions preventing evaporation, with prior addition of cold cyclic AMP (final concentration 2 mM). The tubes were centrifuged and 50 µl of the clear supernatant spotted on Whatmann No.3 MM paper for ascending or descending chromatography, using 1 M ammonium acetate: 95% ethanol (15:35) as a solvent. The chromatograms were run for 18 hours (descending) or for 12 hours (ascending) at room temperature (24 - 25°C), and after drying the cyclic AMP spot was visualized by ultra violet light. The area containing cyclic AMP was cut and counted in 20 ml of Bray's solution in a Packard liquid scintillation spectrometer (Series 3375). The chromatograms also had marker spots for ATP, ADP, AMP, and cyclic AMP. The nonenzymatic formation of cyclic AMP was estimated for every fraction by denaturing the particulate material, followed by the above procedure. The final activity was then calculated after correcting for nonenzymatic cyclic

AMP formation and for quenching. The above method is essentially similar to that described by Drummond and Duncan (119). In some experiments, adenyl cyclase assay was carried out in the above medium, while separation and estimation of cyclic AMP were followed according to the method described by Krishna et al (120). Results obtained by both these procedures were essentially similar. The other conditions and changes whenever employed are described under the respective tables and figures. The protein concentration was determined according to Lowry et al (118). Norepinephrine bitartarate and sodium fluoride were added a few minutes before the starting of the reaction by ATP or fractions.

c) Estimation of Phosphodiesterase Activity

Activity of phosphodiesterase in the homogenates was assayed by measurement of hydrolysis of cyclic-AMP. The assay depends on precipitation of material with high absorbance at 260 mμ , including products of hydrolysis, while cyclic AMP remains in the supernatant fraction. The incubation mixture contained 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$ and 1 mM cyclic AMP in a final volume of 1.0 ml. Reaction was started by addition of homogenate (100 μl) and terminated after 15 minutes of incubation at 37°C by addition of 1 ml of 2% $ZnSO_4$ followed by 1 ml of 1.8% $Ba(OH)_2$. Following centrifugation at 4000 g for 15 minutes, absorbance of the supernatant fraction was measured at 260 mμ, and the rate of degradation of cyclic AMP determined. This method is similar to that described by other investigators (69).

RESULTS

1. Calcium Transport in Subcellular Fractions Isolated from the Hearts of Control and Myopathic Hamsters (210 - 230 day old)

As can be seen from Table I, the hearts of the myopathic hamsters were enlarged (hypertrophic) and the heart wt/body wt ratio in these animals was greater than that of the control ($P < 0.01$). All these myopathic hamsters showed varying degree of pulmonary edema, liver congestion and generalized edema as indications of congestive heart failure. No attempt was made to assess the myocardial function in these animals since an extensive amount of information is available in this regard in the literature (92 - 94). We consider these animals were at the initial stages with a moderate degree of heart failure and were not at the terminal stage which is seen in these hamsters at the age of about 300 days.

Table I also shows that the yield of purified mitochondria and heavy microsomes isolated from the hearts of control and myopathic hamsters was the same. These values of yields do not in any way describe the actual amounts of these subcellular particles in the control and failing hearts. It was also observed that calcium contents of the subcellular fractions obtained from the control and failing hearts were similar (Table II). Once again these values may not reflect the *in vivo* values but provide the required information about the preparations employed in this study for the assessment of results obtained *in vitro* with these fractions. No difference ($P > 0.05$) in the ATP-independent calcium binding of these fractions obtained from the control and failing hearts was noted (Table II). The activities of cytochrome oxidase, 5'-nucleotidase and glucose-6-phosphatase in both mitochondria and heavy microsomes are also reported in Table II. These data on

TABLE I

Heart/Body Weight Ratio and Yield of the Cardiac Subcellular Particles of Control and Myopathic Hamsters

	Control	Myopathic
Age (days)	220 ± 2.5	216 ± 3.1
Heart wt/body wt x 10^3	3.87 ± 0.5	6.16 ± 0.3
Yield of mitochondria (mg protein/g heart wt)	1.48 ± 0.2	1.39 ± 0.3
Yield of reticulum (mg protein/g heart wt)	0.50 ± 0.02	0.45 ± 0.02

The results are a mean ± S.E. of 6 to 10 experiments. The yields of subcellular fractions refer to the amounts of purified particles obtained by the method outlined in the text. The subcellular fractions were isolated by Methods "A" and "B" for calcium transport studies.

TABLE II

Calcium Contents and Marker Enzyme Activities of Mitochondria and Sarcoplasmic Reticulum Isolated from the Control and Failing Hearts

	Mitochondria		Reticulum	
	Control	Failing	Control	Failing
Calcium content (mμmoles/mg protein)	8.3 ± 0.2	8.9 ± 0.4	6.2 ± 0.1	6.1 ± 0.3
ATP independent calcium binding (mμmoles/mg protein)	6.2 ± 0.3	6.0 ± 0.1	5.8 ± 0.3	5.2 + 0.3
Cytochrome oxidase activity	1088 ± 70	1146 ± 54	120 ± 6	130 ± 7
Glucose-6-phosphatase activity	0.13 ± 0.03	0.14 ± 0.02	1.45 ± 0.16	1.58 ± 0.20
5'-Nucleotidase	0.11 ± 0.01	0.14 ± 0.04	1.20 ± 0.15	1.60 ± 0.21

The results are a mean ± S.E. of 4 to 5 experiments. Calcium contents of these fractions were determined by atomic absorption spectrophotometry while ATP independent calcium binding was observed by incubating these fractions for 5 min at 25°C in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 6.8, 0.1 mM $Ca^{45}Cl_2$ and membrane protein (0.2 to 0.3 mg/ml). The methods for cytochrome oxidase, glucose-6-phosphatase and 5'-nucleotidase are described in the text and their activities are expressed as mμmoles cytochrome oxidized/mg protein/min, μmoles Pi/mg protein/hr and μmoles Pi/mg protein/10 min respectively. The subcellular fractions were isolated by Methods "A" and "B" for calcium transport studies.

marker enzymes indicate the degree of purity of the subcellular fractions.

The calcium binding (in the absence of oxalate) by heavy microsomes (Method "A") of the hearts from control and myopathic hamsters was also studied and the results are depicted in Fig. 1. Both the rate and extent of calcium binding by heavy microsomes were less in the failing heart in comparison to the control. It may be mentioned that our values for control calcium binding by hamster heart heavy microsomes are in agreement with those reported in the literature for other species (57, 68, 121). The low calcium binding by heavy microsomes of myopathic hearts could be due to detrimental actions of lysosomal enzymes during preparation since the activities of these enzymes are considered to increase in the failing heart. Therefore acid phosphatase activity of the heavy microsomes was determined using β -glycerol phosphate as substrate by the method of Appelmans et al (122) as described by Katz et al (123) for these particles. The acid phosphatase activity of the control heavy microsomes was 0.1 μmole $\overset{Pi}{\wedge}$ released/mg protein/hr at 37°C and was not different from that of the failing heart. Due to such low acid phosphatase activities in our fractions, it is unlikely that reduced calcium binding by the microsomal fraction of the failing heart could be due to contaminating lysosomes.

Figure 2 shows the calcium uptake (in the presence of 5 mM oxalate) by the heavy microsomes of the control and failing hearts. Although calcium uptake by sarcoplasmic reticulum (Method "A") of the failing heart at 10 minutes of incubation was decreased by about 15% of the control, the values were not significantly different ($P > 0.05$) from each other. The control calcium uptake values of hamster heart microsomal fraction are within the accepted range of values for cardiac microsomes (105, 115, 121, 123 - 125). In some experiments Ca^{++}-stimulated ATP

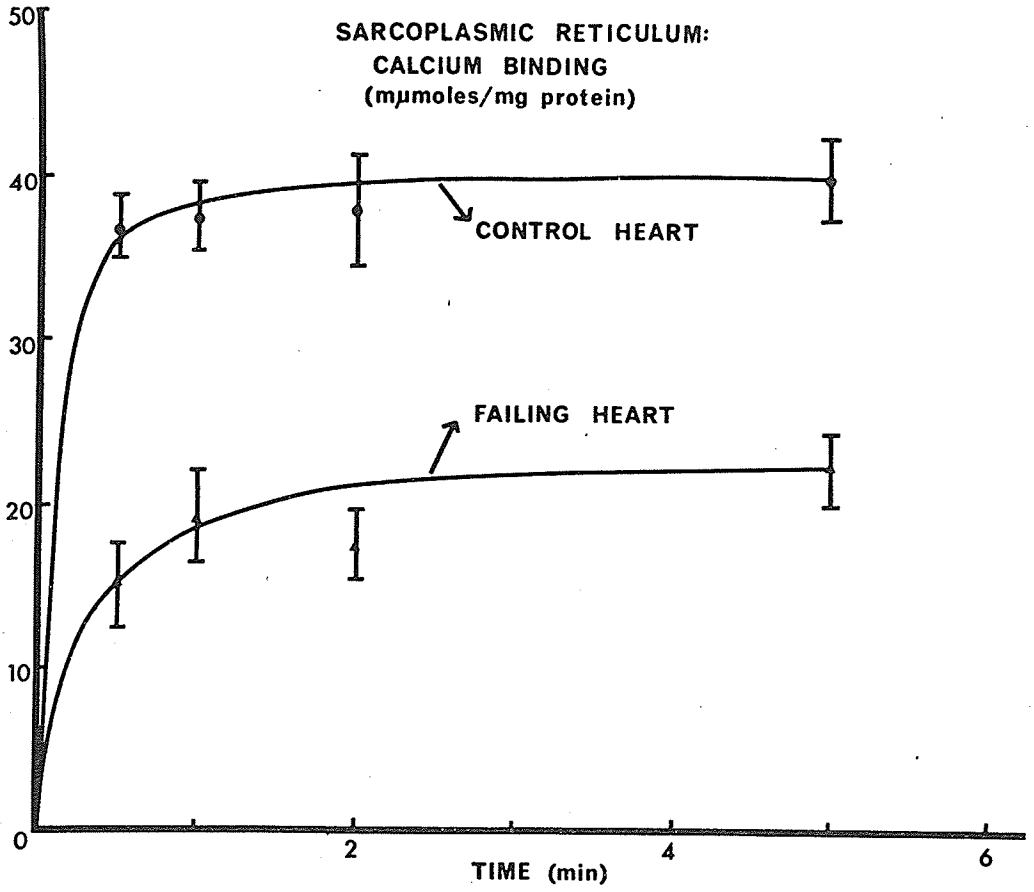

FIGURE 1 Time-course of calcium binding by the heart sarcoplasmic reticulum on incubation in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 6.8, 4 mM ATP and 0.1 mM $Ca^{45}Cl_2$ at 25°C. The protein concentration in the reaction mixture was 0.2 to 0.3 mg/ml. The control hearts were obtained from normal hamsters and the failing hearts from myopathic hamsters (BIO 14.6; 7 to 8 months old). All values are mean ± S.E. of 5 to 6 experiments. The calcium binding by the failing heart reticulum was significantly less than that by the control heart ($P < 0.01$). The reticulum was isolated by method "A" for calcium transport studies.

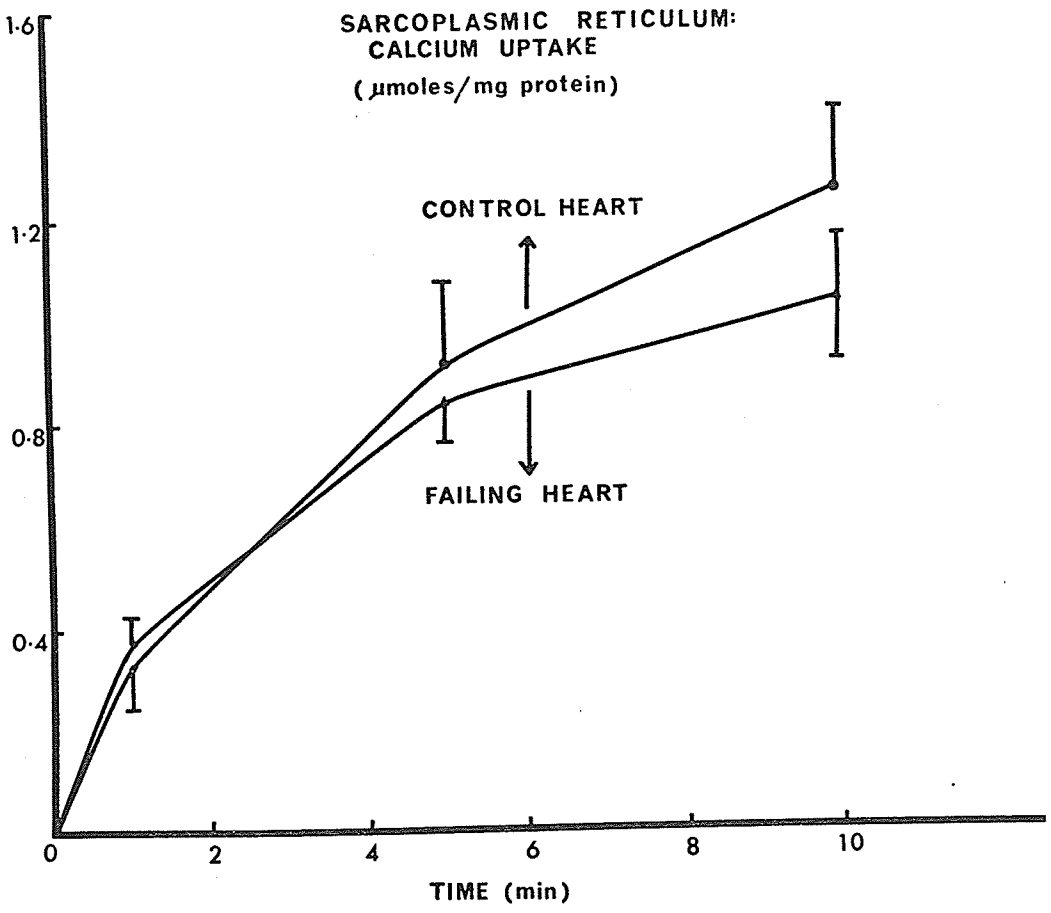

FIGURE 2 Time-course of calcium uptake by the heart sarcoplasmic reticulum on incubation in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 6.8, 4 mM ATP, 0.1 mM $Ca^{45}Cl_2$ and 5 mM potassium oxalate at 37°C. The protein concentration in the reaction mixture was 0.02 to 0.05 mg/ml. The control hearts were obtained from normal hamsters and the failing hearts from myopathic hamsters (BIO 14.6; 7 to 8 months old). All values are mean ± S.E. of 6 experiments. The calcium uptake by the failing heart reticulum at all times of incubation was not significantly different from the control heart ($P > 0.05$). The reticulum was isolated by method "A" for calcium transport studies.

hydrolysis was also measured. Hamster heart microsomal fraction was found to hydrolyze about 0.16 to 0.18 μmoles of ATP/mg protein/min due to the addition of 0.1 mM $CaCl_2$ in the incubation medium containing 100 mM KCl, 10 mM $MgCl_2$, 5 mM potassium oxalate, 20 mM Tris-HCl, pH 6.8, 4 mM ATP and 0.03 mg/ml membrane protein at 37°C. The values for Ca^{++}-stimulated ATPase of the failing heart heavy microsomes were not different from those for the control heart.

Both calcium binding (in the absence of Pi and succinate) and calcium uptake (in the presence of 4 mM Pi and 5 mM succinate) were studied in mitochondria obtained from the control and failing hearts (Method "B"). The results described in Fig.3 and Fig.4 indicate a marked reduction in calcium accumulating ability of the mitochondria isolated from the hearts of myopathic animals. The control values for calcium binding and calcium uptake by hamster heart mitochondria are within the same range as described for other species (56, 62, 66, 68).

In another series of experiments calcium binding activities of the subcellular fractions isolated by Methods "A" and "B" from the control and failing hearts were also determined in the absence or presence of oligomycin and sodium azide, the two well known inhibitors of the ATP supported calcium pump of mitochondria. The results shown in Table III indicate that calcium binding by heavy microsomes was unaffected by both oligomycin and sodium azide while that of mitochondria was inhibited by 50 to 60% of the respective values for the control and failing hearts. Inability of azide and oligomycin to influence calcium accumulation by heavy microsomes has also been reported by other workers (57, 68, 126, 127). The subcellular fractions isolated from the control and failing hearts after 20 seconds of homogenization were found to bind the same amount of calcium as that observed for these

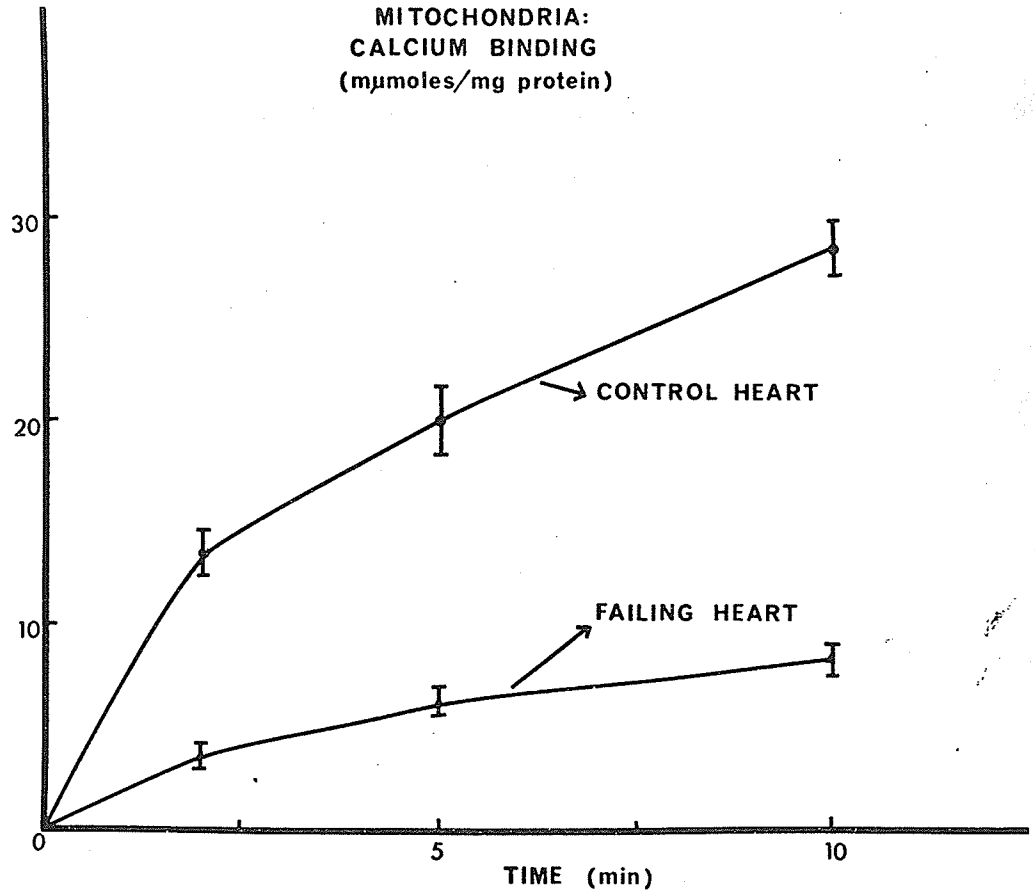

FIGURE 3 Time-course of calcium binding by the heart mitochondria on incubation in a medium containing 100 mM KCl, 20 mM Tris-HCl, pH 6.8, 4 mM ATP, 10 mM $MgCl_2$ and 0.1 mM $Ca^{45}Cl_2$ at 25°C. The protein concentration in the reaction mixture was 0.3 to 0.5 mg/ml. The control hearts were obtained from normal hamsters and the failing hearts from myopathic hamsters (BIO 14.6; 7 to 8 months old). All values are mean ± S.E. of 4 experiments. The calcium binding by the failing heart was significantly less than that by the control heart ($P < 0.01$). The mitochondria were isolated by method "B" for calcium transport studies.

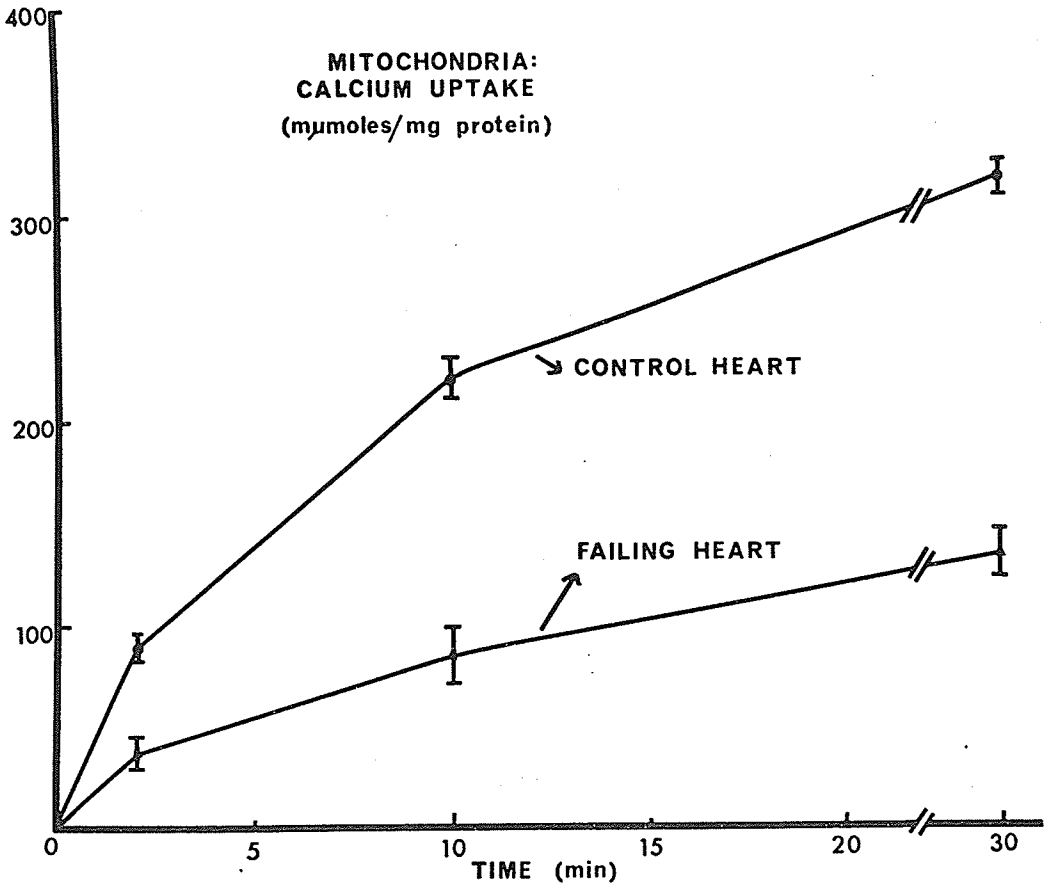

FIGURE 4 Time-course of calcium uptake by the heart mitochondria on incubation in a medium containing 100 mM KCl, 20 mM Tris-HCl, pH 6.8, 4 mM ATP, 10 mM $MgCl_2$, 0.1 mM $Ca^{45}Cl_2$, 4 mM Pi and 5 mM sodium succinate at 37°C. The protein concentration in the reaction mixture was 0.2 to 0.3 mg/ml. The control hearts were obtained from normal hamsters and the failing hearts from myopathic hamsters (BIO 14.6; 7 to 8 months old). All values are mean ± S.E. of 5 experiments. The calcium uptake by the failing heart was significantly less than for the control heart ($P < 0.01$). The mitochondria were isolated by method "B" for calcium transport studies.

TABLE III

Influence of Oligomycin and Azide on the Calcium Binding Activities of Sarcoplasmic Reticulum and Mitochondria Isolated from Control and Failing Hearts

Additions	Calcium Binding (% of values without inhibitors)			
	Sarcoplasmic Reticulum		Mitochondria	
	Control	Failing	Control	Failing
-	100	100	100	100
Oligomycin (2.5 μg/ml)	93 ± 3.6	95 ± 2.5	58 ± 4.2	62 ± 3.6
Sodium Azide (5 mM)	98 ± 1.2	94 ± 2.4	50 ± 3.8	61 ± 4.4

The results are a mean ± S.E. of 3 experiments. The values of calcium binding in the absence of inhibitors by sarcoplasmic reticulum of the control and failing hearts were 45 ± 2.8 and 20 ± 3.5 mμmoles/mg protein respectively at 5 min of incubation, while these values for mitochondria of the control and failing hearts were 36 ± 4.0 and 11 ± 1.2 mμmoles/mg protein respectively at 10 min of incubation. Both sarcoplasmic reticulum (0.20 mg protein/ml) and mitochondria (0.25 mg protein/ml) were incubated at 25°C in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 4 mM Na-ATP, 20 mM Tris-HCl, pH 6.8 and 0.1 mM $Ca^{45}Cl_2$. The inhibitors were added 2 min before starting the reaction by ATP and the reaction was terminated by millipore filtration at the times indicated above. The sarcoplasmic reticulum was isolated by procedure "A" and mitochondria by procedure "B" for calcium transport studies.

hearts after 45 seconds of homogenization.

ATP hydrolyzing activities of microsomal and mitochondrial fractions isolated by Methods "A" and "B" respectively from the control and failing hearts were also studied in the absence or presence of oligomycin and sodium azide. The data in Table IV show that total ATPase activities of both heavy microsomes and mitochondria of the control hearts were not different from those of the failing heart ($P > 0.05$). It may be noted that both oligomycin and sodium azide produced inhibition of total ATPase activities of the subcellular fractions. Preliminary experiments in this laboratory showed no effect of azide on the extra ATP-split by reticulum due to 0.1 mM Ca^{++}. These observations concerning the effect of azide and oligomycin on total ATPase activity of heavy microsomes are in agreement with earlier reports (57, 68, 123, 126, 127).

Since we were unable to observe significant differences between the calcium uptake (in the presence of oxalate) by heavy microsomes of the control and failing hearts, we thought to study both calcium binding and uptake by heavy microsomes isolated by a technique different than that described under Method "A". The microsomal fraction, isolated by the Method "C", was equally active in terms of calcium binding but showed a lesser activity for calcium uptake than that obtained by the Method "A". The data reported in Table V reveal a decrease in calcium binding without any appreciable differences between calcium uptake by heavy microsomes of the control and failing hearts. These results clearly show a defect in calcium accumulation (in the absence of oxalate) by the cardiac heavy microsomes of myopathic hamsters. The total ATPase activity (Ca^{++} - Mg^{++} stimulated) was also determined in the subcellular fractions obtained by Method "C" from the

TABLE IV

Influence of Oligomycin and Azide on the ATPase Activities of Sarcoplasmc Reticulum and Mitochondria Isolated from Control and Failing Hearts

Additions	ATPase Activity (μmoles Pi released/mg protein)			
	Control	Failing	Control	Failing
A. Heavy Microsomes	2 min. of incubation		5 min. of incubation	
-	4.05 ± 0.23	4.10 ± 0.32	7.62 ± 0.65	6.53 ± 0.47
Oligomycin (2.5 μg/ml)	3.33 ± 0.44	3.46 ± 0.36	6.19 ± 0.42	5.82 ± 0.39
Sodium Azide (5 mM)	2.81 ± 0.29	2.62 ± 0.33	3.75 ± 0.46	3.25 ± 0.26
B. Mitochondria	5 min. of incubation		10 min. of incubation	
-	3.33 ± 0.19	3.53 ± 0.16	5.66 ± 0.25	5.25 ± 0.33
Oligomycin (2.5 μg/ml)	2.18 ± 0.24	2.32 ± 0.20	3.41 ± 0.30	3.04 ± 0.24
Sodium Azide (5 mM)	1.66 ± 0.15	1.52 ± 0.18	2.58 ± 0.28	2.72 ± 0.31

The results are a mean ± S.E. of 3 experiments. The sarcoplasmic reticulum and mitochondria were isolated according to the method "A" and "B" respectively for calcium transport studies. These subcellular fractions (0.20 to 0.25 mg protein/ml) were incubated at 25°C in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 4 mM Na-ATP, 20 mM Tris-HCl, pH 6.8 and 0.1 mM $CaCl_2$. The inhibitors were added 2 min before starting the reaction by ATP and the amount of Pi present in the protein-free filtrate was measured at the times indicated in this Table.

TABLE V

Calcium Accumulation in the Absence or Presence of Oxalate by Sarcoplasmic Reticulum Isolated from the Control and Failing Hearts

	Calcium Accumulation	
	Control	Failing
	(mμmoles/mg protein)	
A. Calcium binding (no oxalate) at 5 min	36.40 ± 4.98	19.78 ± 4.85
B. Calcium uptake (5 mM oxalate) at 1 min	265 ± 31	189 ± 53
5 min	330 ± 52	337 ± 62
10 min	431 ± 81	480 ± 87

The results are a mean ± S.E. of 6 experiments. The subcellular particles were incubated in the medium containing 100 mM KCl, 10 mM $MgCl_2$, 4 mM ATP, 0.1 mM $CaCl_2$, 20 mM Tris-HCl, pH 6.8. The reaction was started by the addition of subcellular particles to give a final concentration of protein (0.2 to 0.3 mg/ml) for binding and 0.05 to 0.07 mg/ml for uptake studies. These particles were isolated according to the method "C" for calcium transport studies.

control and failing hearts and the results are reported in Table VI. No difference in the ATPase activity of mitochondria or heavy microsomes was noted between the control and failing hearts. The high initial rate of ATP hydrolysis by the hamster heart subcellular fractions is similar to that for the rat heart reported earlier (56, 68).

In order to test whether the defect in calcium accumulation by mitochondria is only limited to heart, the mitochondria of liver were isolated according to the Method "D" described in the Method Section. The data in Table VII shows a decrease ($P < 0.01$) in calcium binding (in the absence of Pi and succinate) but no difference ($P > 0.05$) in calcium uptake (in the presence of 4 mM Pi and 5 mM succinate) by the liver mitochondria from the myopathic hamsters in comparison to the control. Thus it appears that in 220 day old myopathic hamsters (BIO 14.6) heart mitochondria are more susceptible and reveal a greater degree of damage to calcium transport mechanism than liver mitochondria or cardiac heavy microsomes.

Further experiments were carried out in order to test whether the observed reduction in calcium transport by the failing heart mitochondria was due to changes in the affinity of membrane sites to bind calcium or in the rate of reaction. The results shown in Fig. 5 and Fig. 6 indicate no difference in the affinity constants (50 μM) of the control and failing heart mitochondrial membranes to bind calcium in the absence or presence of 5 mM Pi. The V_{max} values in the absence of Pi were 250 and 166 nmoles Ca^{++}/mg protein and in the presence of 5 mM Pi were 660 and 500 nmoles Ca^{++}/mg protein for the control and failing heart mitochondria respectively. The reduction in calcium accumulating ability of the failing heart mitochondria was also seen when various concentrations of Pi

TABLE VI

ATPase Activity of Heavy Microsomes and Mitochondria Isolated from Control and Failing Hearts

Time of Incubation (min)	ATPase Activity (μmoles Pi released/mg protein)			
	Heavy Microsomes		Mitochondria	
	Control	Failing	Control	Failing
1	3.33 ± 0.22	3.87 ± 0.31	2.94 ± 0.44	2.27 ± 0.28
5	6.62 ± 0.07	5.77 ± 0.77	5.82 ± 0.82	5.63 ± 0.32
10	8.93 ± 0.43	7.37 ± 1.82	8.15 ± 0.90	8.05 ± 1.01

The results are a mean ± S.E. of 5 experiments. The subcellular fractions were incubated in the medium containing 100 mM KCl, 10 mM $MgCl_2$, 4 mM ATP, 0.1 mM $CaCl_2$, 20 mM Tris-HCl, pH 6.8 at 37°C. The reaction was started by the addition of subcellular particles to give a final concentration of protein (0.2 to 0.3 mg/ml). These particles were isolated according to the method "C" for calcium transport studies.

TABLE VII

Calcium Accumulation by Liver Mitochondria Isolated from Control and Myopathic Hamsters

	Calcium Accumulation (mμmoles/mg protein)	
	Control	Myopathic
Calcium binding (in the absence of Pi and succinate)	120.87 ± 2.85	66.96 ± 1.5
Calcium uptake (in the presence of 4 mM Pi and 5 mM succinate)	147.30 ± 3.0	141.37 ± 1.0

The results are a mean ± S.E. of 4 experiments. The mitochondria were incubated for 5 min in a medium containing 100 mM KCl, 20 mM Tris-HCl, pH 6.8, 10 mM $MgCl_2$, 4 mM ATP, 0.1 mM $Ca^{45}Cl_2$ at a protein concentration of 0.2 to 0.3 mg/ml. For experiments on calcium uptake (37°C) 4 mM Pi and 5 mM sodium succinate were also present in the incubation medium while these were absent when calcium binding was determined at 25°C. The mitochondria were isolated by Method "D" for calcium transport studies.

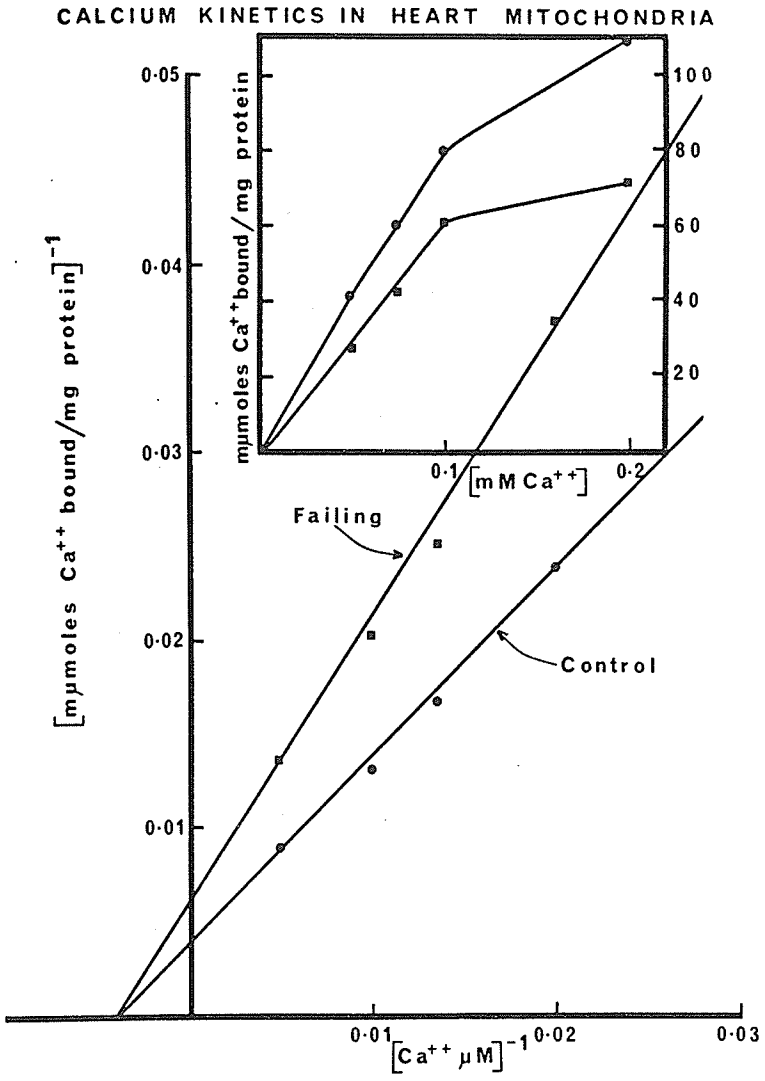

FIGURE 5

Mitochondria isolated from control and failing hearts (Method B) were incubated in the standard incubation medium in the presence of indicated concentrations of calcium. The reaction stopped by millipore filtration as described in "Methods". The reciprocal plots of calcium binding versus calcium concentration revealed that affinity constants for calcium of the mitochondrial membranes of control and failing hearts were not different. However, V_{max} of failing mitochondrial preparation was markedly reduced. The results shown are taken from a representative experiment. Similar results were observed for at least 3 separate preparations.

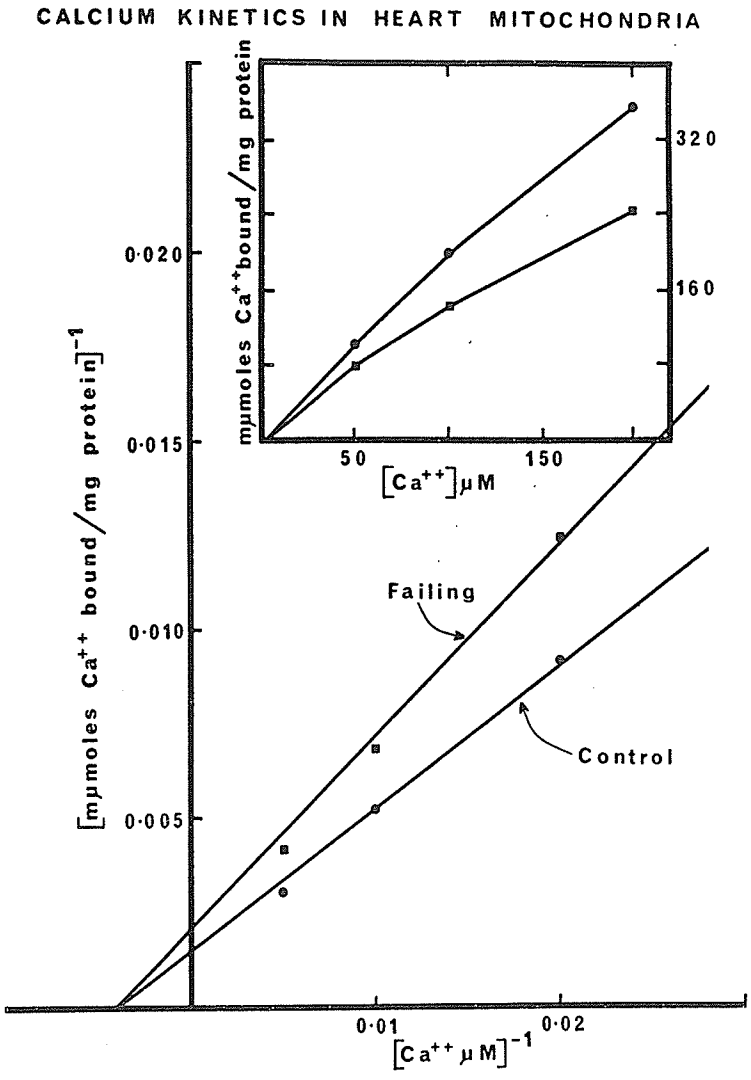

FIGURE 6 Calcium accumulation in the presence of Pi by control and failing heart mitochondria at different calcium concentrations. Incubation media same as that described under Fig. 4. Please note that affinity for calcium of failing heart mitochondria is not different when compared to control, although V_{max} is markedly reduced. Results of a representative experiment shown. Similar results were obtained with at least 3 preparations.

were present in the incubation medium (Fig. 7).

2. Calcium Transport in Heavy Microsomal Fraction Isolated from the Hearts of Control and Myopathic Hamsters (260 - 275 day old)

In view of our inability to demonstrate an abnormality in calcium uptake by the heavy microsomal fraction in the hearts of 210 - 230 day old myopathic hamsters, it was considered necessary to use animals at advanced stage of heart failure (260 - 275 day old animals). The microsomal fractions from the hearts of these animals were incubated in media of different pH and the calcium binding by the membranes of failing hearts was found to be markedly decreased (Fig. 8). The results shown in Fig. 9 and Fig. 10 indicate a reduction in the ability of heavy microsomes isolated from the failing hearts to accumulate calcium in the presence of varying amounts of Pi or oxalate. These experiments reveal an impairment of calcium uptake by the cardiac heavy microsomal fraction at the later stages of heart failure.

The calcium uptake in the presence of 5 mM oxalate by the heart microsomal fractions was also studied in the presence of varying amounts of K^+ or Na^+ in the incubation medium. The results in Fig. 11 reveal that increasing the concentrations of K^+ or Na^+ in the medium resulted in an increase in calcium transport. This is essentially in agreement with the results reported by Katz and Repke (125) for the dog heart microsomes. The optimal concentrations of K^+ or Na^+ in the incubation medium were in the range of 80 to 100 mM. It can also be seen from Fig. 11 that calcium accumulating ability of the microsomal fraction was less in the failing heart in comparison to the control at each concentration of K^+ or Na^+ employed in this experiment.

Since Mg^{++} is essential for the energy-dependent calcium uptake by the

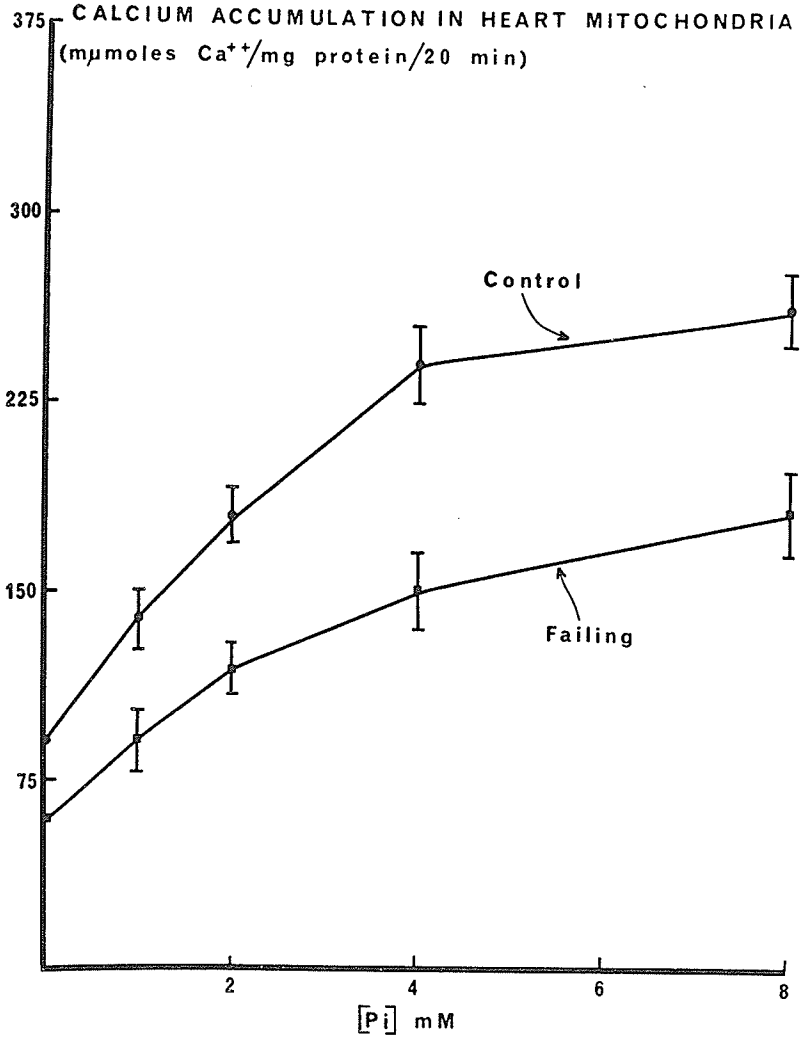

FIGURE 7 Calcium accumulation by control and failing heart mitochondria in the presence of different concentrations of Pi. Reaction mixture same as described in Fig. 4 except for the indicated amounts of Pi. Please note that at all Pi concentrations tested failing heart mitochondria accumulated reduced amount of calcium ($P < 0.05$). Each value is mean ± S.E. of at least 4 experiments using separate preparations.

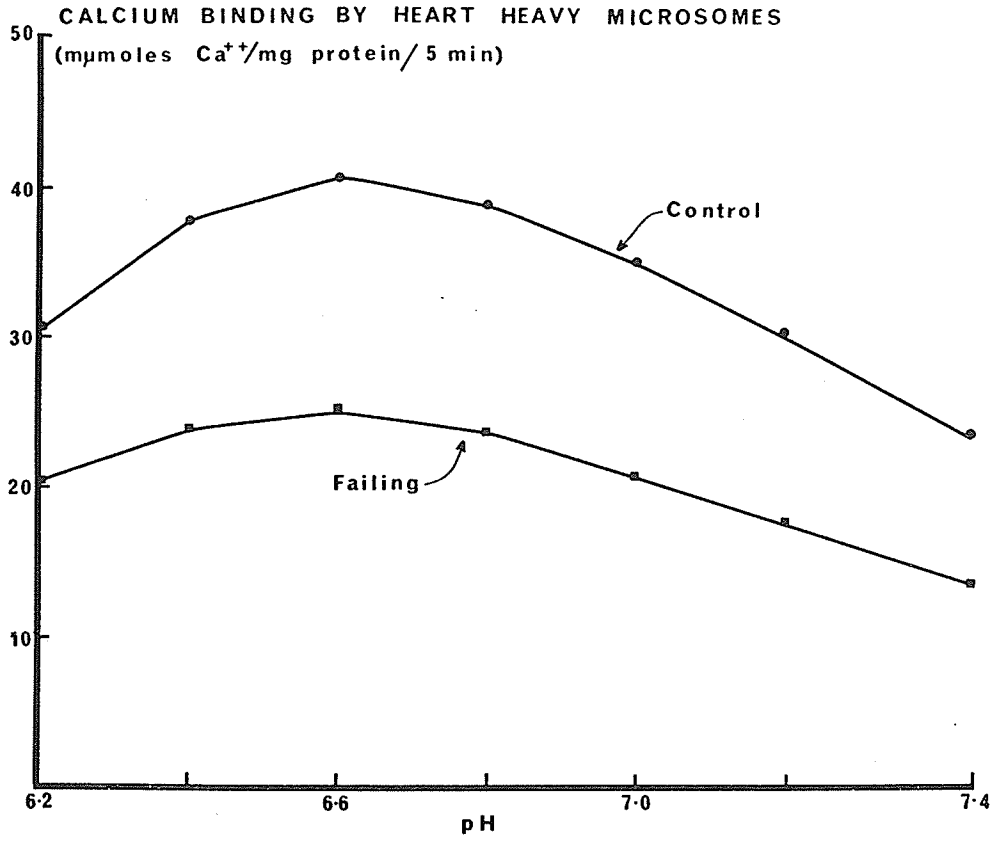

FIGURE 8 Calcium binding at different pH by cardiac heavy microsomal fractions isolated from control and myopathic hamsters (260 - 275 day old). The conditions for the incubation were similar to those described under Figure 1 except Tris-maleate buffer was used instead of Tris-HCl. At all pH tested, myopathic heart vesicles bound significantly less calcium per mg protein. Protein concentration - 0.30 mg/ml for control and 0.34 mg/ml for myopathic microsomes. Results of one representative experiment are shown. Similar results were observed with 5 such preparations.

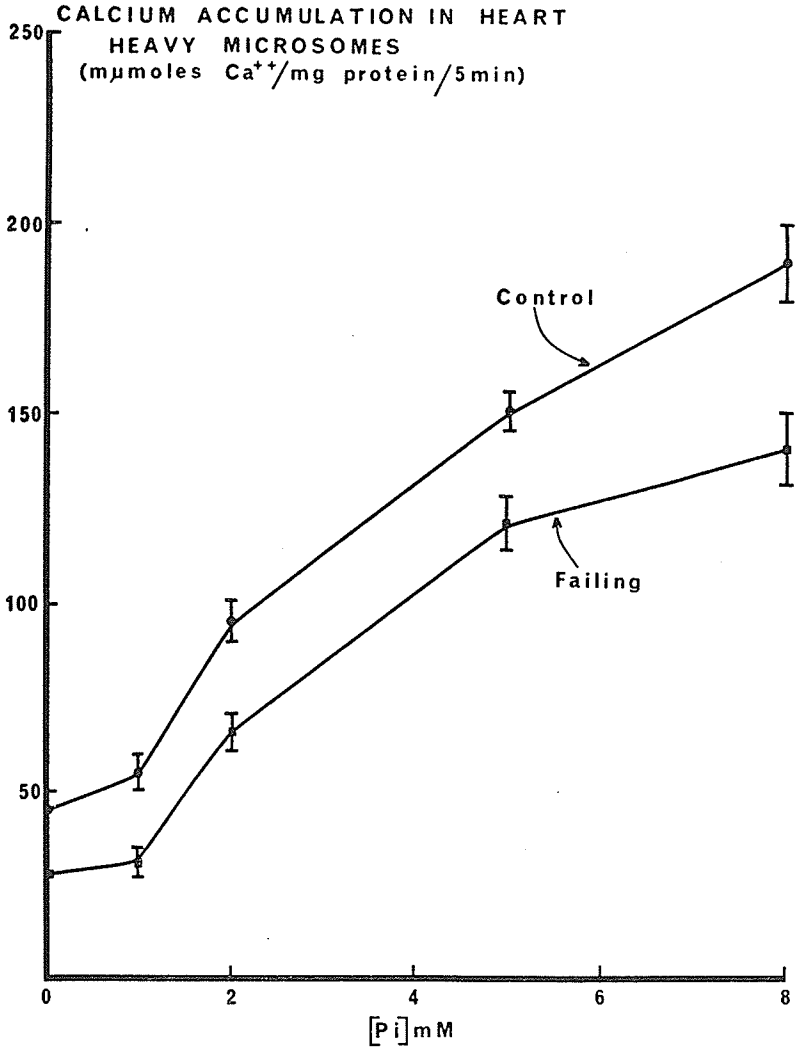

FIGURE 9 Calcium accumulations by heart microsomes in the presence of different concentrations of Pi. Incubation medium contained 100 mM KCl, 20 mM Tris-HCl, pH 6.8, 4 mM ATP, 10 mM $MgCl_2$, and vesicular protein 0.25 - 0.40 mg/ml with indicated amounts of Pi. Each value is mean ± S.E. of 6 determinations carried out with 4 separate microsomal preparations isolated from control and failing hearts of hamsters (260 - 275 day old). In the absence or presence of varying amounts of Pi, myopathic heart vesicles accumulated reduced amount of calcium per mg protein ($P < 0.05$).

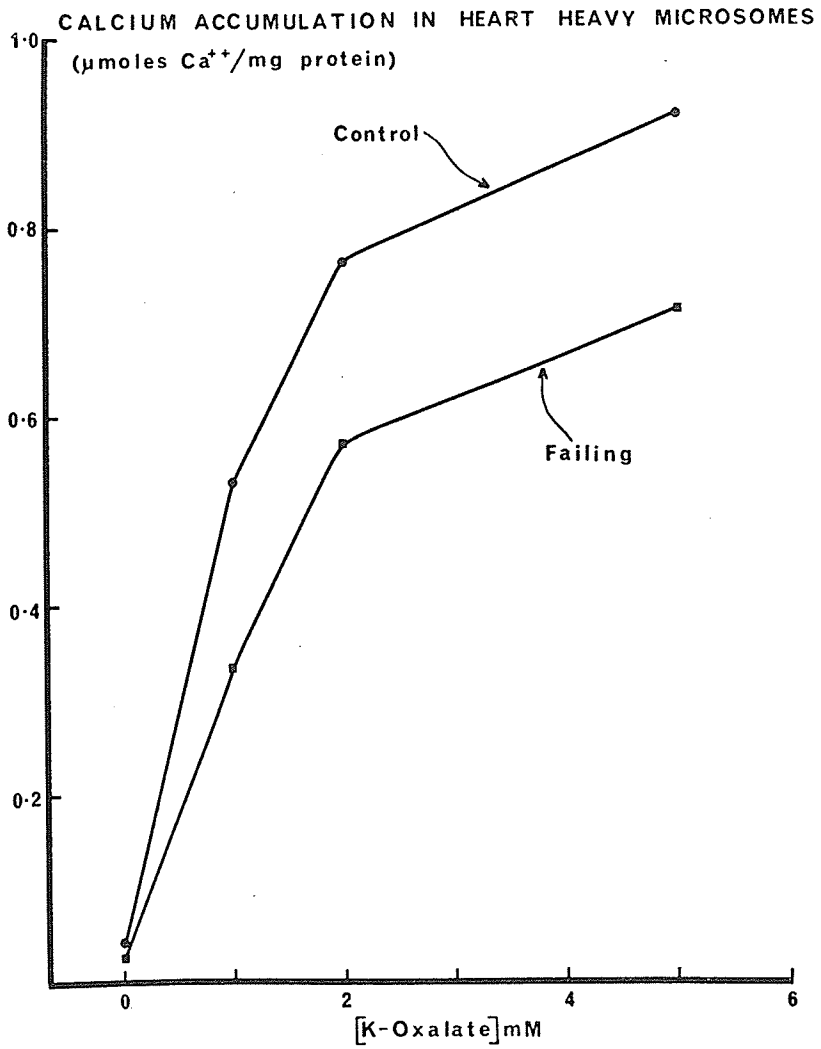

FIGURE 10 Calcium accumulation by cardiac heavy microsomes in the presence of different concentrations of oxalate. Control and myopathic hamsters were 260 - 275 day old. All other conditions are similar to those described under Figure 2 except that varying amounts of oxalate were present. Time of incubation - 10 min at 37°C. Protein concentrations for control and failing heart microsomes were 0.05 mg/ml and 0.03 mg/ml respectively. Results from one representative experiment are shown. Similar results were obtained with 4 such preparations. At all concentrations of K-oxalate, failing cardiac vesicles accumulated reduced amount of calcium ($P < 0.01$).

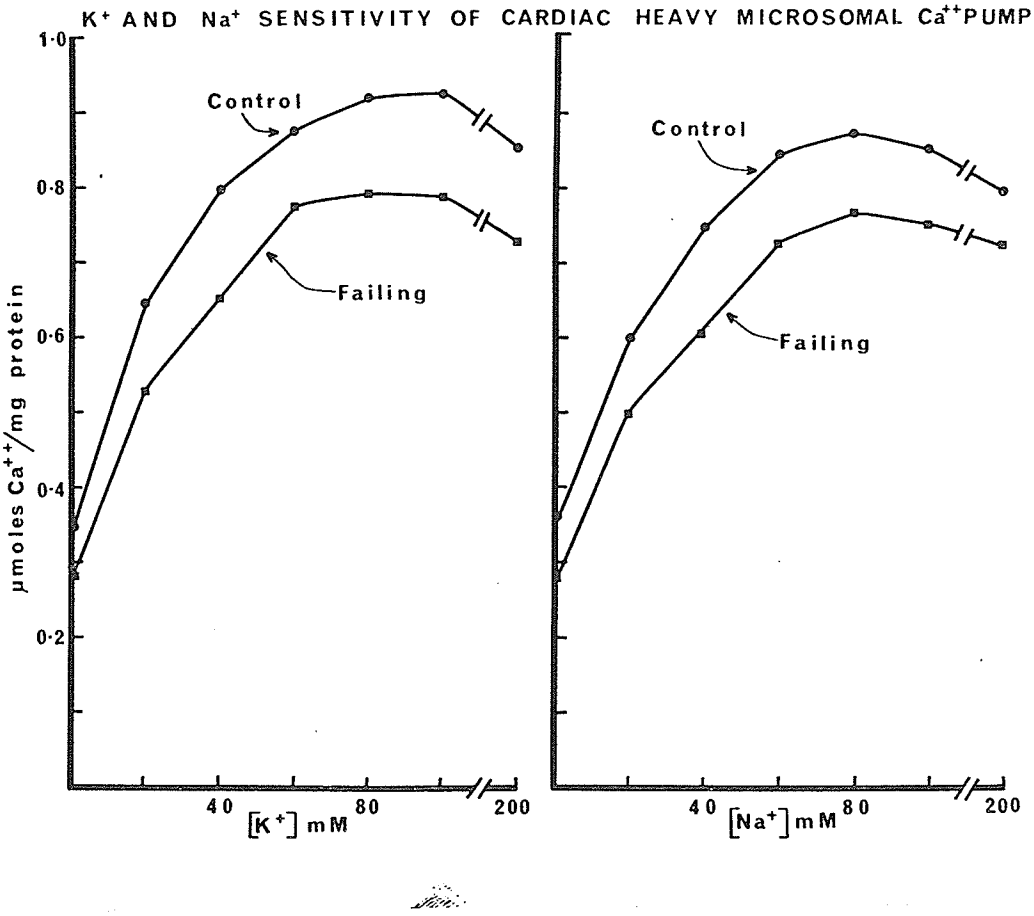

FIGURE 11 K^+ and Na^+ sensitivity of cardiac heavy microsomal Ca^{++} pump. Conditions similar to those described under Figure 2 except for varying amounts of KCl and NaCl. Control and myopathic hamsters, 260 - 275 day old were employed. Time of incubation - 10 min at 37°C. Protein concentrations were 0.062 and 0.053 mg/ml for the control and failing microsomes respectively. The results obtained from one typical experiment are shown. At each salt concentration the fai ling heart vesicles accumulated significantly reduced amount of calcium per mg of protein.

sarcotubular vesicles, experiments were carried out for determining calcium accumulation by the control and failing heart heavy microsomes in the presence of different concentrations of this cation. Increasing the concentration of Mg^{++} from 1 μM to 1 - 2 mM in the medium was found to increase the calcium uptake by heart microsomes (Fig. 12). At low concentrations of Mg^{++} (< 100 μM) no appreciable difference in calcium accumulation was noted between the control and failing heart microsomes. However, at concentrations of Mg^{++} higher than 100 μM, failing heart microsomes were observed to accumulate significantly lesser amounts of Ca^{++} in comparison to the control ($P < 0.05$). Analysis of these results according to Lineweaver-Burk (128) revealed that two types of binding sites, low affinity and high affinity, for Mg^{++} are located in these membranes (Fig. 13). In the failing heart there appears to be no change in the high affinity site for Mg^{++}. At saturating Mg^{++} concentrations, the affinity for Mg^{++} (low affinity) was increased in the failing heart microsomes in comparison to the control; however, the V_{max} was decreased.

The calcium uptake by cardiac microsomes was also studied in the presence of various concentrations of ATP. Increasing the concentration of ATP was found to increase the calcium uptake by both control and failing heart microsomes (Fig. 14); however, failing heart microsomes accumulated less calcium than the control at each concentration of ATP. Lineweaver-Burk plots of these results (Fig.15) indicated two types of affinity sites for ATP (low and high affinity sites). No change in the affinity for ATP at low affinity sites (K_m = 160 μM) was noted, although the values for V_{max} of the reactions were 166 and 123 mμmoles Ca^{++}/ mg protein/min for the control and failing heart microsomes respectively. Both

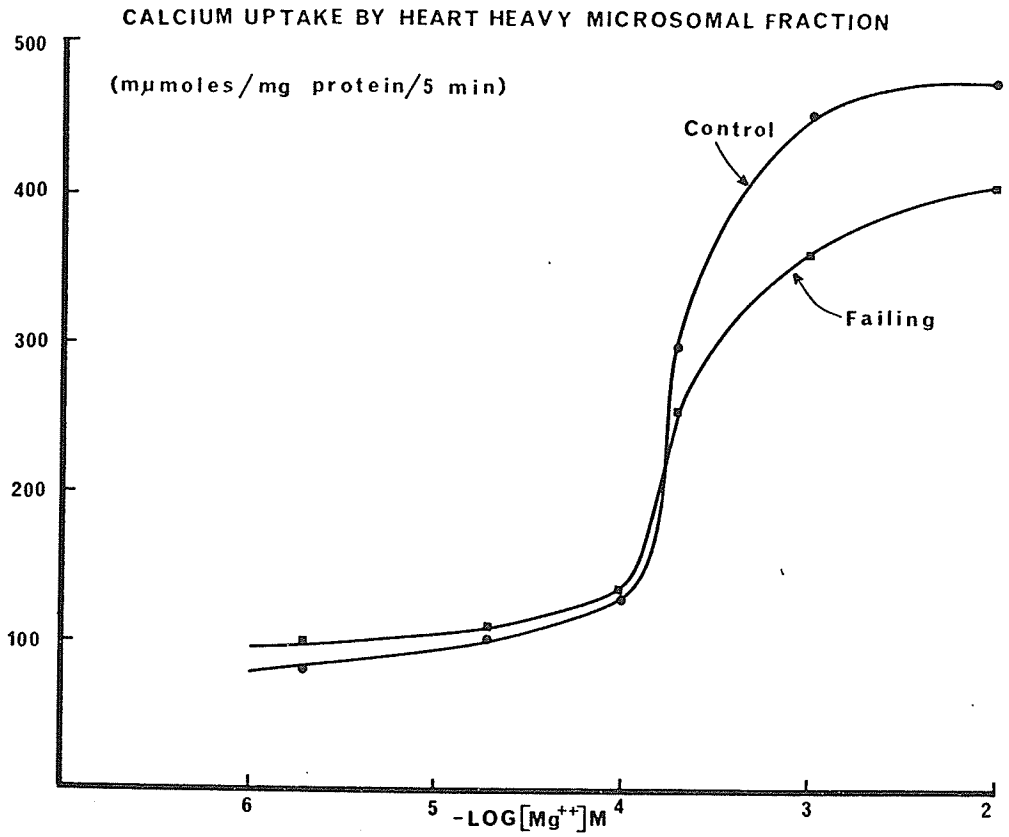

FIGURE 12 Calcium uptake by vesicles isolated from control and failing hamster hearts (260 - 275 day old) in the presence of varying amounts of $MgCl_2$. At lower $MgCl_2$ concentrations (less than 100 μM) control and failing heart vesicles accumulated similar amounts of calcium; however, at concentrations higher than 100 μM the vesicles from failing heart accumulated reduced amounts of calcium per mg protein. Other conditions were similar to those described under Figure 2. Protein concentrations were 0.039 mg and 0.052 mg/ml for control and failing cardiac vesicles respectively. The time of incubation was 5 min at 37°C. Similar results were observed with 3 separate vesicular preparations.

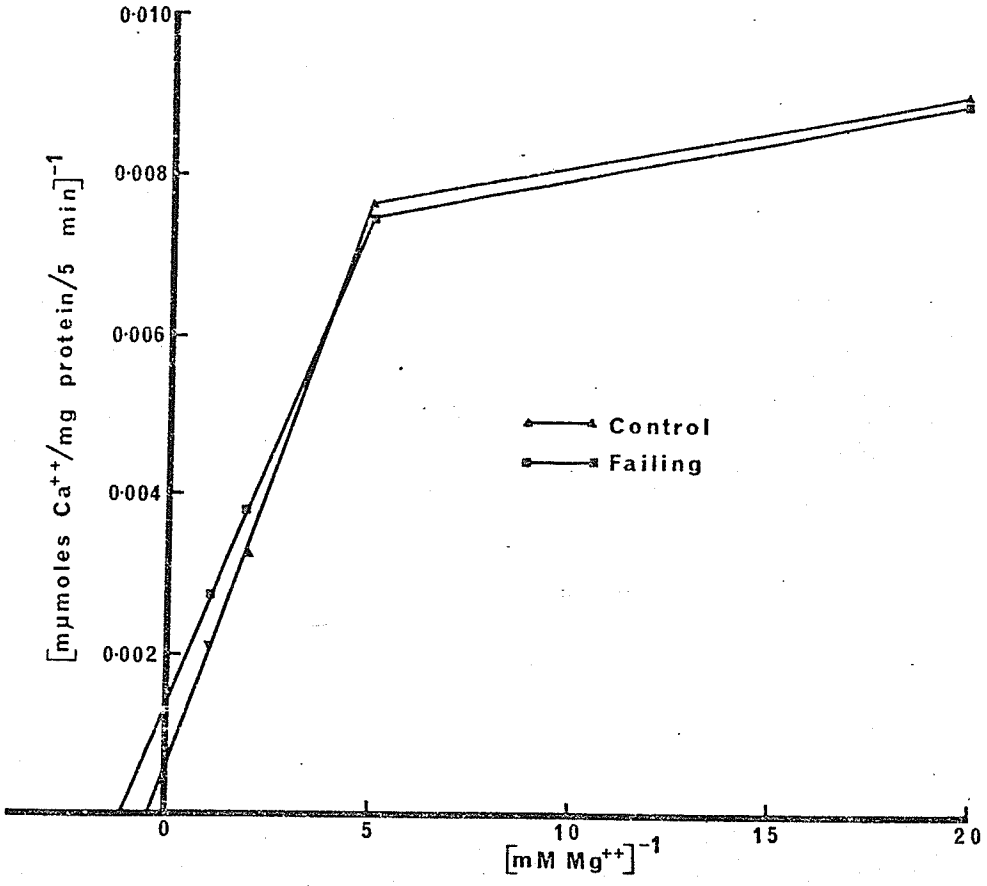

FIGURE 13 Lineweaver-Burk plot of the results described in Figure 12.

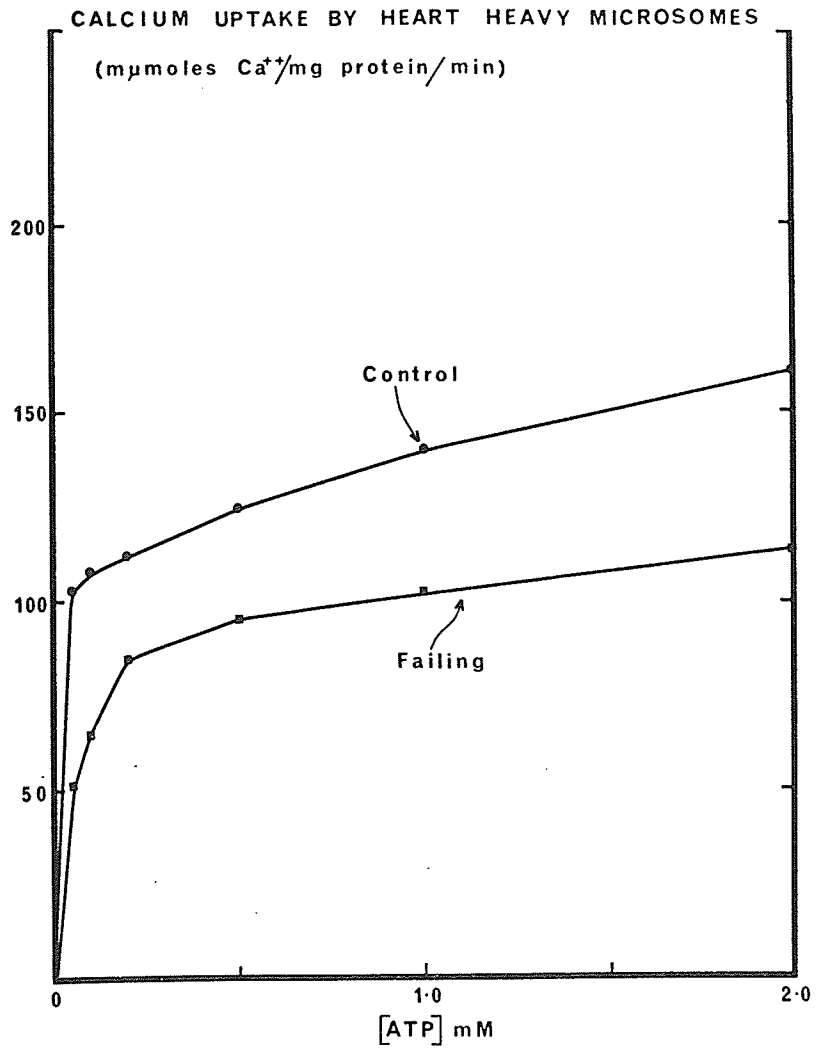

FIGURE 14 Effect of varying concentrations of ATP on the calcium uptake by vesicles prepared from control and failing hearts of hamsters (260 - 275 day old). All conditions were similar to those described under Figure 2 except for ATP concentrations. The time of incubation was 2 min at 37°C. Protein concentration was 0.058 mg/ml for the control and failing heart microsomes. Similar results in addition to the one described above were obtained with 3 separate such preparations. At each ATP concentration, failing heart vesicles have reduced rate of calcium accumulation.

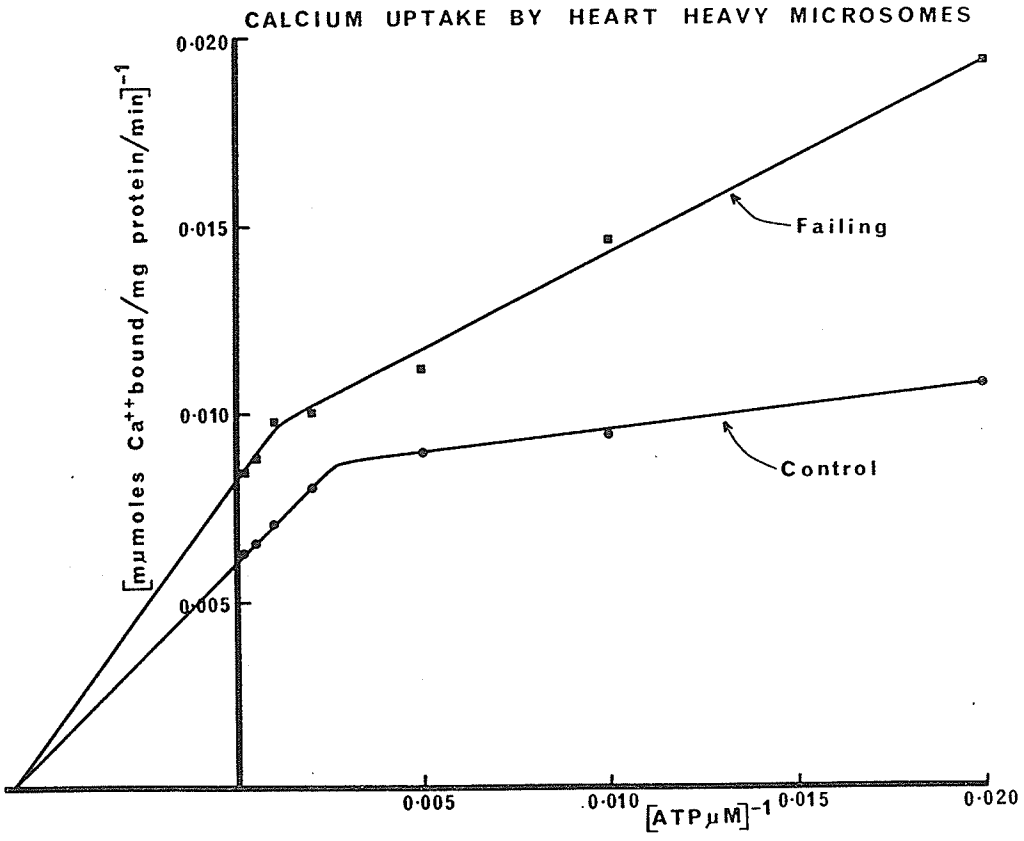

FIGURE 15 Lineweaver-Burk plot of the data described in Figure 14. At high ATP concentrations, K_m values were the same for the control and failing cardiac heavy microsomes. However, at lower ATP concentrations, the K_m value for the failing heart vesicles was greater than that of the control. At both higher and lower concentrations of ATP, V_{max} of the reaction was markedly reduced for failing heart vesicles. Similar results were seen with 3 other microsomal preparations. Results suggest alterations in the high affinity sites and not in the low affinity sites in the failing heart heavy microsomal membranes.

high affinity constants and V_{max} of the reactions at low concentrations of ATP were decreased in the failing heart microsomes.

The kinetics of calcium transport by the control and failing hearts microsomes was further studied in the absence or presence of 5 mM oxalate and the results are shown in Fig. 16 and Fig. 17. Increasing the concentrations of calcium was found to increase calcium accumulation by heart microsomes both in the absence and presence of oxalate. The calcium binding and transporting abilities of the failing heart microsomes were less than the control values. Lineweaver-Burk plot of these data did not show any change in the affinity of the failing heart microsomes to bind calcium (K_a = 25 μM). The values for V_{max} of the reactions in the absence of oxalate were 77 and 48 mμmoles Ca^{++}/mg protein (Fig. 16) whereas these values in the presence of oxalate were 425 and 298 mμmoles Ca^{++}/mg protein/min (Fig. 17) for the control and failing heart microsomes respectively. These results indicate an impairment in the "calcium pump" mechanism in the hearts of myopathic hamsters at advanced stages of heart failure.

3. $Na^+ - K^+$ ATPase Activities of Control and Failing Hearts

The activities of $Na^+ - K^+$ ATPase and Mg^{++} ATPase of the fractions obtained during the isolation procedure from the control and myopathic hamster (220 - 230 day old) hearts are shown in Table VIII. The Mg^{++} ATPase activities of the fractions obtained after deoxycholate as well as NaI treatments from the control hearts were not different from those of the myopathic hearts ($P > 0.05$). On the other hand, $Na^+ - K^+$ ATPase activities of both these fractions from the myopathic hearts were markedly higher than those of the control. The activity of $Na^+ - K^+$ ATPase from the control hearts is comparable to the values reported

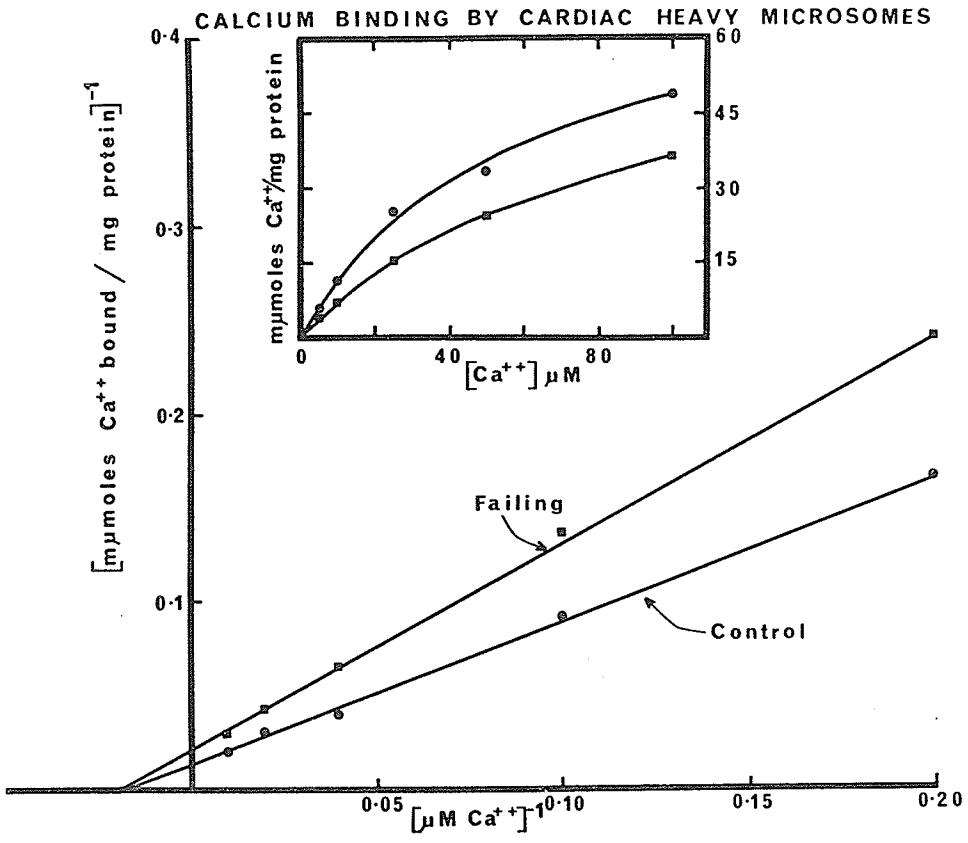

FIGURE 16 Kinetics of calcium binding by cardiac heavy microsomal fractions isolated from control and failing hearts of hamsters (260 - 275 day old). Conditions were similar to those described under Figure 1. At each calcium concentration (5 - 100 μM), failing heart vesicles bound a reduced amount of calcium in comparison to the control. From the results it can be seen that the affinity (25 μM) for calcium of the membrane sites is unaffected although V_{max} is markedly reduced for failing cardiac vesicles. Similar results obtained with 5 such preparations.

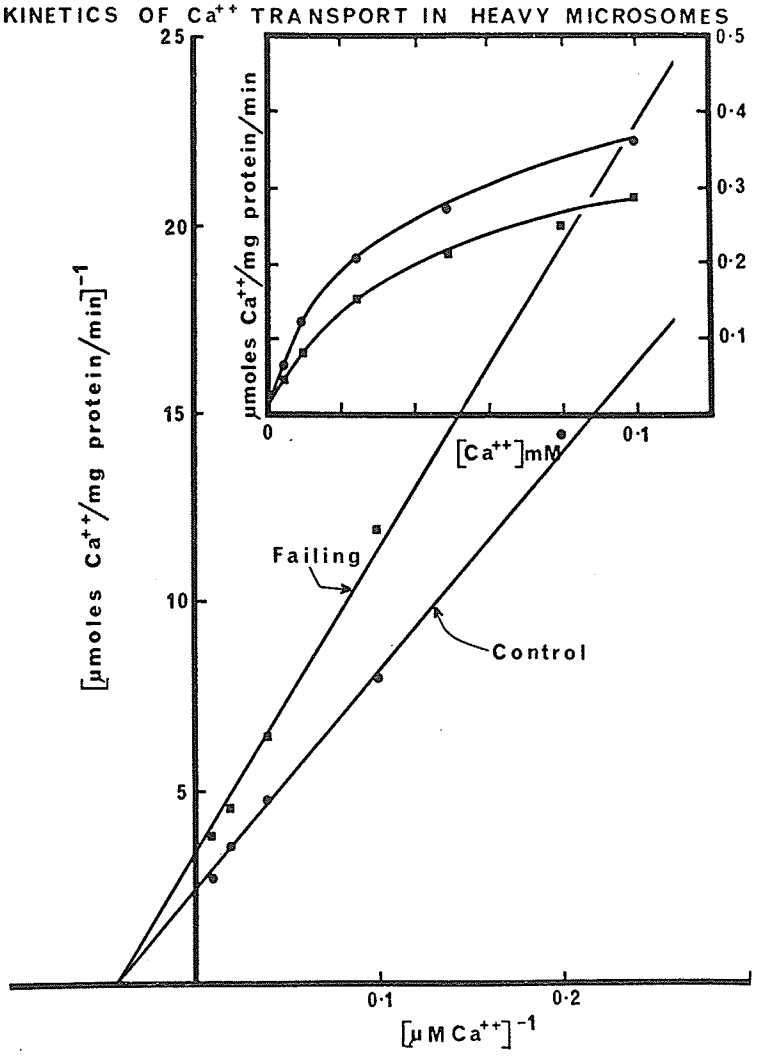

FIGURE 17 Kinetics of calcium uptake in the presence of 5 mM oxalate by cardiac heavy microsomes. The conditions were similar to those under Figure 16 except that oxalate (5 mM) was present. Membrane protein concentration was 0.043 mg/ml and the temperature was 37°C. Failing heart vesicles accumulated reduced amount of calcium at each calcium concentration (5 - 100 µM).

TABLE VIII

Mg^{++}ATPase and Na^{+}-K^{+} ATPase Activities of Hearts of Control and Myopathic Male Hamsters (210 - 230 day old)

Fractions	ATPase Activity			
	Method A		Method B	
	Control	Myopathic	Control	Myopathic
	μmoles Pi/mg protein/hr			
A. Deoxycholate Treated				
Mg^{++}ATPase	18.9 ± 2.2	23.5 ± 2.5	17.5 ± 2.8	20.8 ± 2.4
Na^{+}-K^{+}ATPase	14.5 ± 1.3	26.3 ± 2.1	16.0 ± 1.4	37.2 ± 2.2
B. NaI Treated				
Mg^{++}ATPase	10.6 ± 1.2	13.2 ± 2.3	9.8 ± 1.7	13.5 ± 2.5
Na^{+}-K^{+}ATPase	22.5 ± 1.5	39.8 ± 1.6	24.2 ± 2.3	44.0 ± 2.4

The hearts of control and myopathic hamsters were homogenized according to Method "A" and "B" (see "Method" section) and the fractions isolated after deoxycholate and sodium iodide treatment were assayed for the enzyme activities immediately in the medium containing 50 mM Tris-HCl, pH 7.4 - 7.6, 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 1 mM EDTA, 4 mM Tris-ATP at a protein concentration of 40 - 60 μg/ml at 37°C for 10 min. Please note that myopathic heart Na^{+}-K^{+} ATPase activity is significantly higher than that of the control at both steps of the isolation procedure ($P < 0.05$). Each value is a mean ± S.E. of 6 experiments.

by other investigators (129, 130, 131). All the subsequent experiments on Na^+ - K^+ ATPase activity were carried out with NaI-treated preparations. It can be seen from Fig. 18 that the Na^+ - K^+ ATPase activities for both the control and myopathic hearts were linear with respect to the time of incubation as well as the protein concentration employed in this study.

The pH optima for Na^+ - K^+ ATPases of the control and failing hearts were about 7.4 to 7.6. The data shown in Fig. 19 demonstrates that Na^+ - K^+ ATPase activity of the myopathic heart was significantly higher ($P < 0.01$) than that of the control at each pH tested in these experiments. In another series of experiments the concentrations of Na^+ and K^+ were varied simultaneously while the osmolarity of the incubation medium was kept constant. The results described in Fig. 20 reveal the optimal activating concentrations of Na^+ and K^+ were about 100 and 20 mM respectively both for the control and myopathic heart enzymes. Furthermore, the Na^+ - K^+ ATPase activity of the failing heart was markedly higher than the control at every Na^+ : K^+ ratio.

The Na^+ - K^+ ATPase activities of the control and myopathic hearts were also studied by varying the amounts of Mg ATP (1:1) in the incubation medium. The optimum concentration of Mg ATP was about 2 mM in both cases. The substrate velocity curves (Fig. 21) were sigmoidal in shape and the Hill plots (132) of the data revealed n values > 1 for both control and failing heart enzymes. It can be seen again that the activity of the myopathic heart ATPase was higher than the control under the experimental conditions employed in this series.

The kinetic properties of Na^+ - K^+ ATPases from the control and failing hearts were further studied by changing the concentrations of either K^+, while

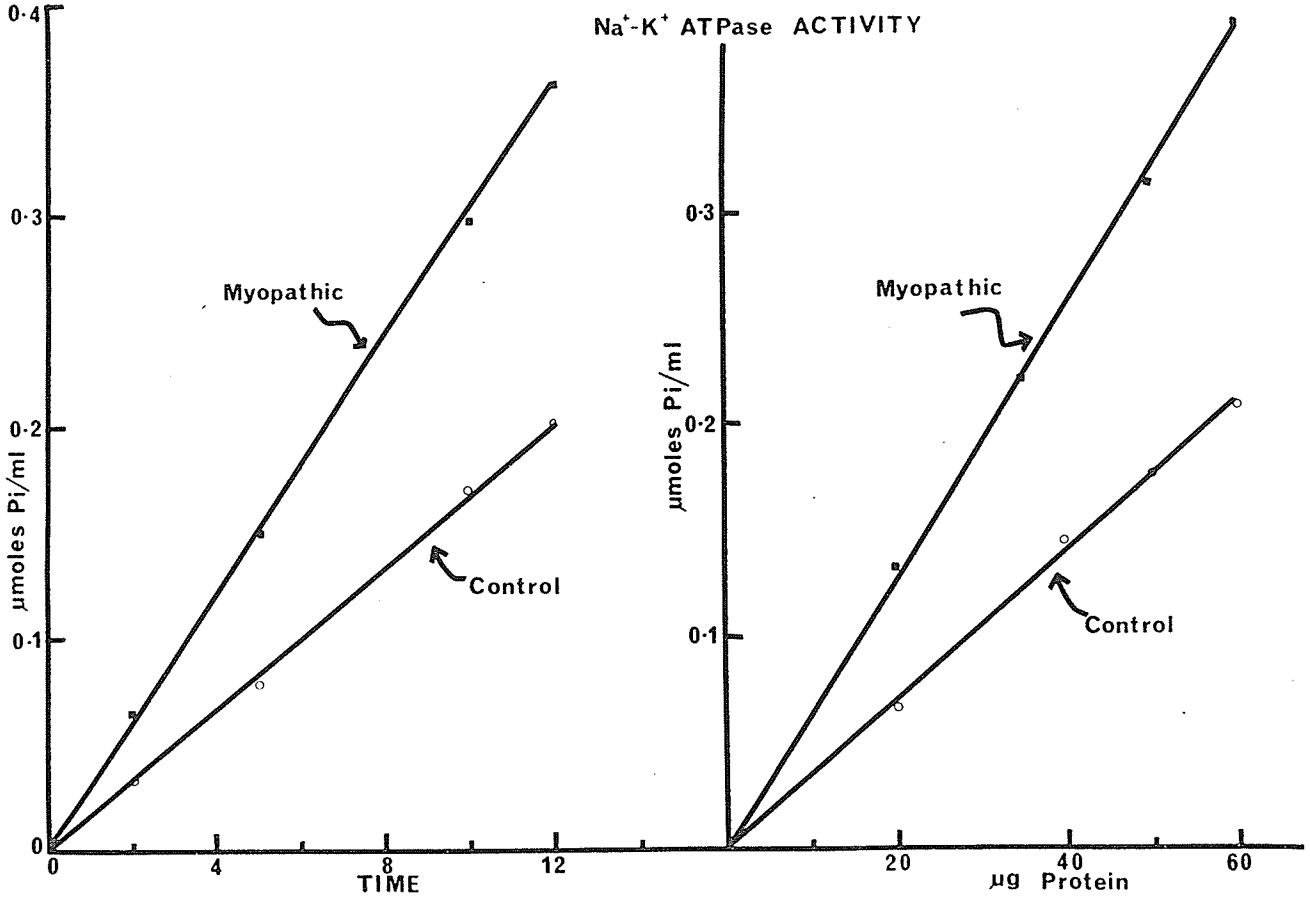

FIGURE 18 $Na^+ - K^+$ ATPase activities of control and failing heart fractions (NaI treated). The results show the linearity of the enzymatic system with respect to time of incubation and protein concentration. Please note that failing heart fractions had higher enzymatic activity in comparison to control. The subsequent experiments were performed using protein concentration less than 60 µg/ml of incubation medium and incubation time of 10 minutes.

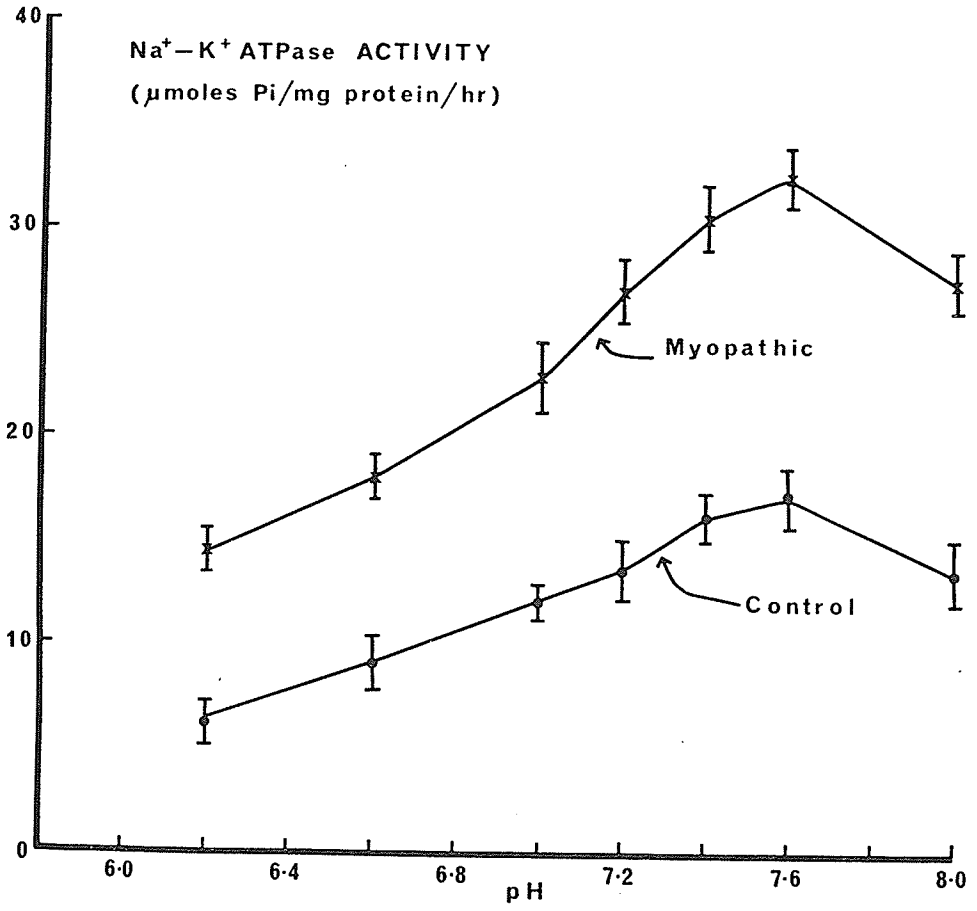

FIGURE 19

Relation of pH and enzyme activity. The incubation medium contained 50 mM Tris-HCl or 50 mM Tris-maleate of respective pH's, 1 mM EDTA, 5 mM $MgCl_2$, 4 mM Tris-ATP, about 40 to 60 μg protein/ml. The fractions incubated for 10 minutes at 37°C in the above medium in the presence or absence of 100 mM NaCl and 20 mM KCl. The difference is referred to as $Na^+ - K^+$ ATPase. Please note that enzyme has relatively broad pH optima (7.4 to 7.8). At each pH, the failing heart fractions had greater activity. Each point is mean ± S.E. of at least 3 separate preparations.

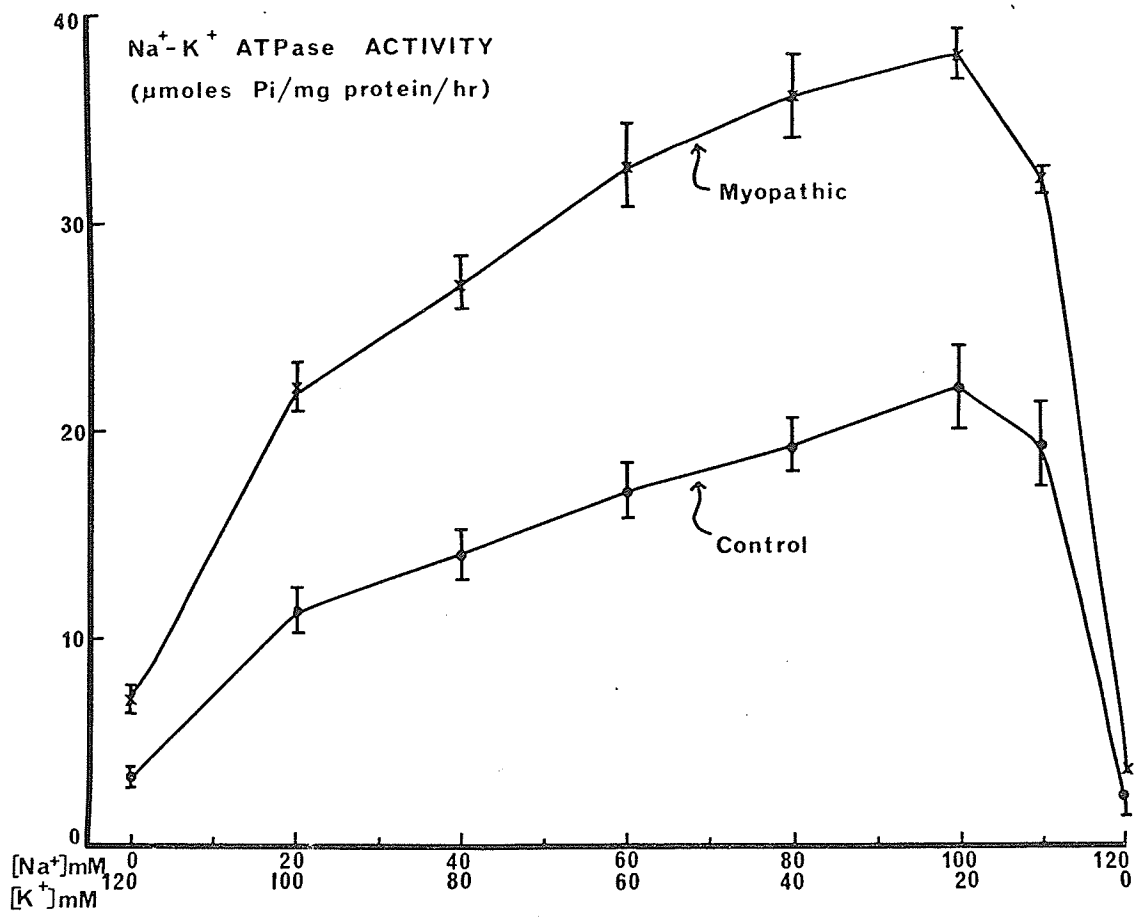

FIGURE 20 Na^+:K^+ ratio and Na^+ - K^+ ATPase activity. The enzyme activity of the NaI-treated fractions isolated from control and myopathic (BIO 14.6, 210 - 230 day old) was assayed in the presence of different concentrations of Na^+ and K^+ while keeping Na^+ + K^+ concentration constant. All other conditions similar to those described under "Method". Please note that failing heart fractions possessed higher enzyme activities in comparison with control (n = 3).

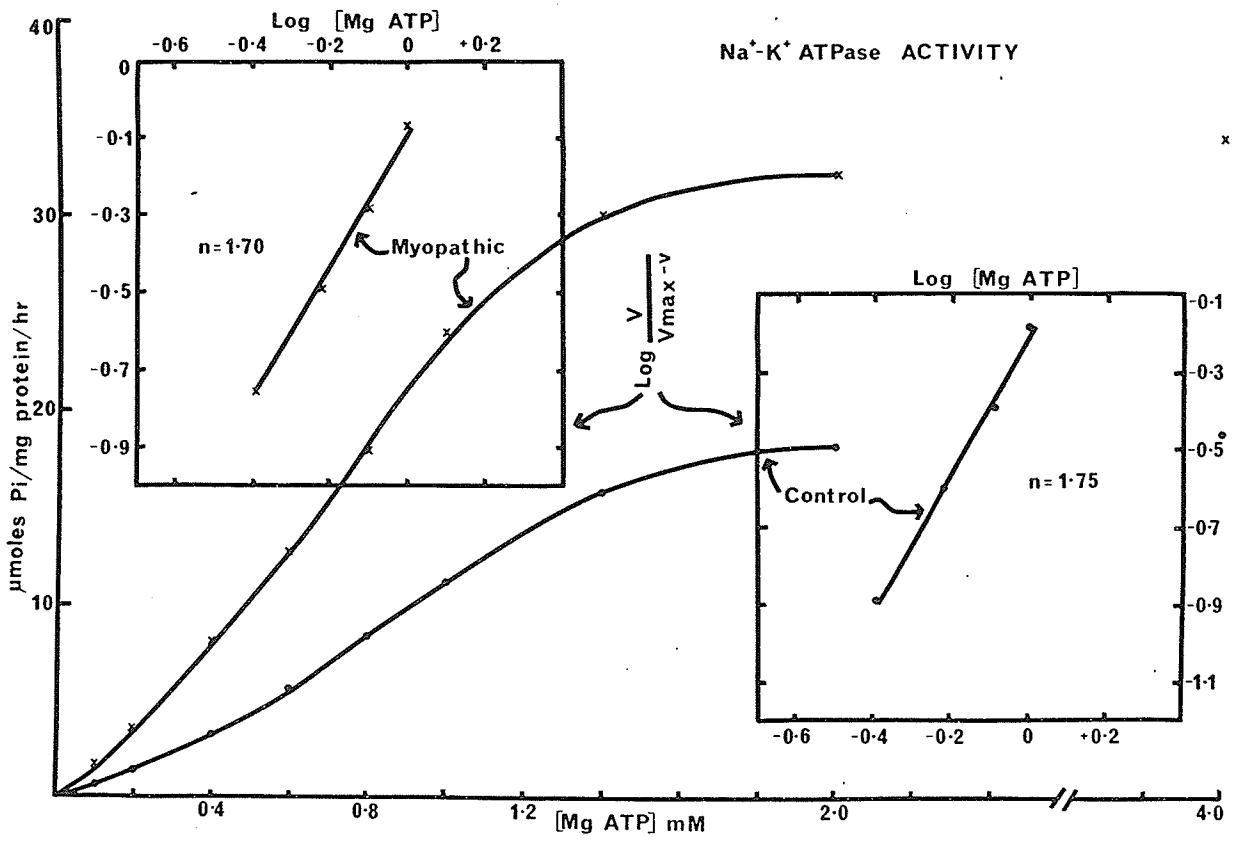

FIGURE 21 Saturation kinetics with MgATP (substrate). NaI-treated fractions of control and failing hearts were incubated in the standard assay medium for 10 minutes at 37°C with the indicated amounts of MgATP (Mg:ATP = 1). Results obtained from a typical experiment. Similar results were observed for at least 3 separate preparations. The enzyme isolated from control and failing hearts is allosteric with respect to substrate activation. Although K_m, & values are similar V_{max} for failing heart preparations is markedly higher (also see Table IX).

keeping Na^+ (100 mM) constant, or by changing Na^+, while keeping K^+ (20 mM) constant. The results are shown in Fig. 22 and Fig. 23. Under these conditions, it may be noted that the reaction velocity curves were sigmoidal in shape and the activity of the myopathic heart enzyme was higher than the control. The Hill plots of the data showed values for $n > 1$ for both control and failing heart Na^+ - K^+ ATPases.

The kinetic parameters for both control and failing heart Na^+ - K^+ ATPase preparations are described in Table IX. It is apparent that the value of the V_{max} for the enzyme of the myopathic heart is higher ($P < 0.001$) than the control, while K_m values for these enzymes were not different from each other. It can be also noted that the n values for Mg ATP, Na^+, or K^+ of the failing heart enzyme are comparable to the control.

Both ouabain and calcium are known to inhibit Na^+ - K^+ ATPase activity of myocardium (82, 83). The inhibitory effects of various concentrations of ouabain and calcium were tested on enzyme preparations isolated from both the control and myopathic hamster hearts and the results are shown in Fig. 24 and Fig. 25. No difference in the inhibitory responses of the Na^+ - K^+ ATPases isolated from the control and failing hearts to either ouabain or calcium was observed. The ouabain sensitivity of the enzyme preparations is comparable to reported values for heart Na^+ - K^+ ATPase of other species (130, 131).

4. Adenyl Cyclase Activities of Control and Failing Hearts

The adenyl cyclase activities of both control and failing heart homogenates were determined in the presence of ATP regenerating system. The rates of reactions were found linear with respect to time and protein concentration employed

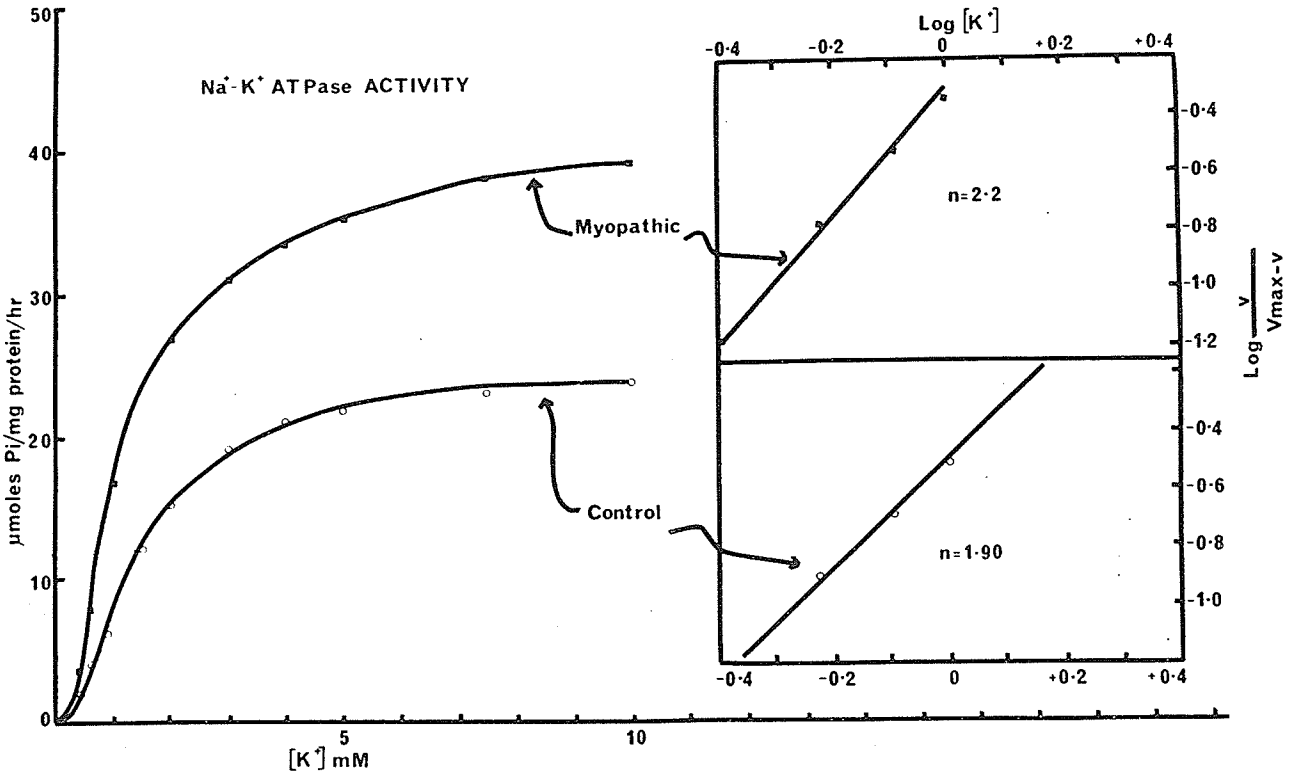

FIGURE 22 Response to K^+ ion concentrations. Enzyme preparations of control and failing hearts incubated in the standard assay medium except the concentrations of K^+ were as indicated. Results of a typical experiment. Similar results were seen with other preparations. Please note the S-shape of the activity curve and homotropic co-operativity of interaction. The n values were greater than 1 for all the preparations tested. However, no difference in the n values, obtained for control and failing heart enzyme preparations, was observed (See Table IX).

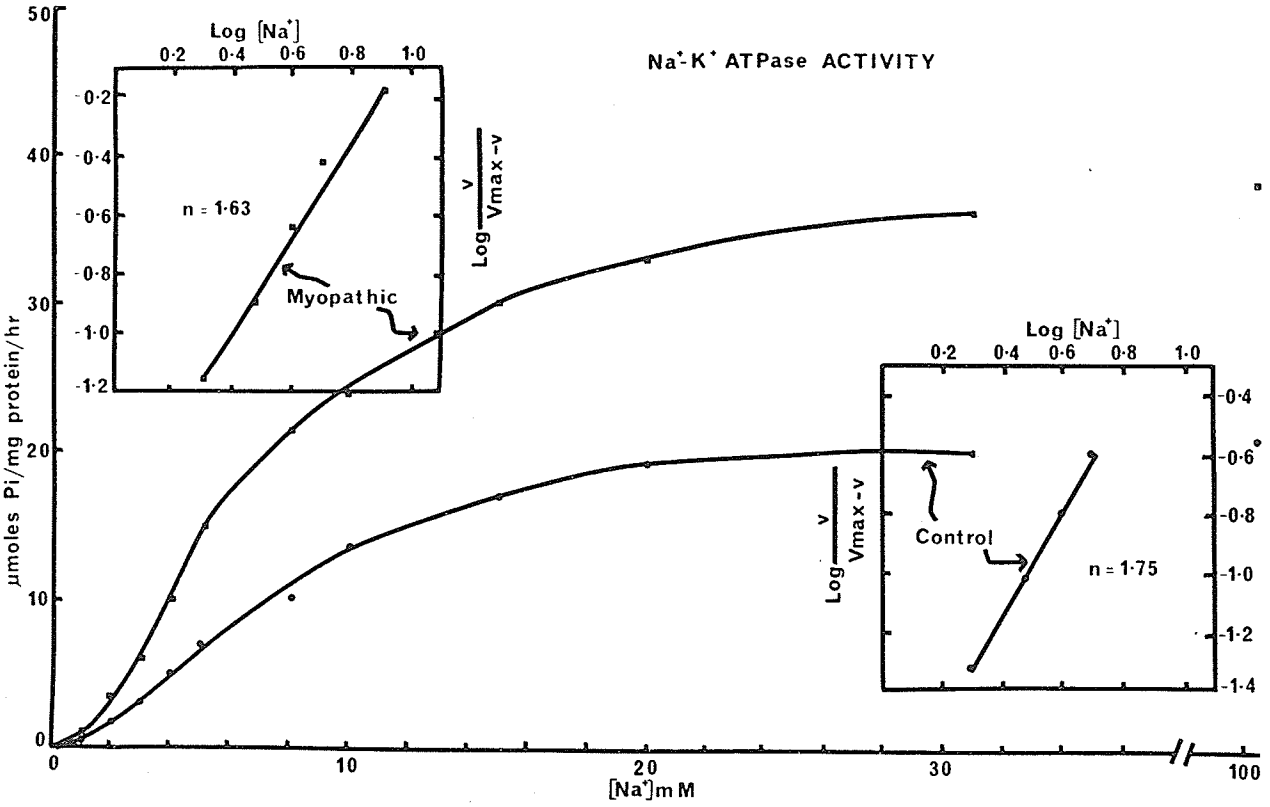

FIGURE 23 Response to Na^+ ion concentrations. Conditions similar as described in "Methods" except Na^+ ion concentration as indicated above. It can be seen that enzymes isolated from control and failing heart exhibits allsoteric characteristics. (See Table IX).

TABLE IX

Kinetic Parameters of Na^+-K^+ ATPases Isolated from Hearts of Control and Myopathic Male Hamsters (210 - 230 day old)

	Control	Myopathic
V_{max} (μmoles Pi/mg protein/hr)	33.50 ± 2.29	59.16 ± 2.48
K_m (mM ATP)	0.73 ± 0.04	0.79 ± 0.04
n value (Mg ATP)	1.57 ± 0.05	1.56 ± 0.05
n value (Na^+)	1.58 ± 0.05	1.61 ± 0.07
n value (K^+)	1.65 ± 0.10	1.87 ± 0.11
Ki_{50} (Ouabain, x 10^{-7}M)	5.70 ± 1.86	4.40 ± 0.69

Sodium-iodide treated Na^+-K^+ ATPase preparations isolated from control and failing hamster hearts were employed for kinetic analysis. Lineweaver-Burke analysis was used to obtain K_m (ATP) values and n values were calculated from the Hill plots of the data. Each value is a mean ± S.E. of at least 3 separate preparations. Please note that K_m (ATP) and n values for MgATP, Na^+ or K^+ observed for failing heart enzyme are not different than that for control; however, V_{max} of the activity is significantly greater. Enzymes from both the control and failing hearts exhibited similar sensitivity to ouabain inhibition.

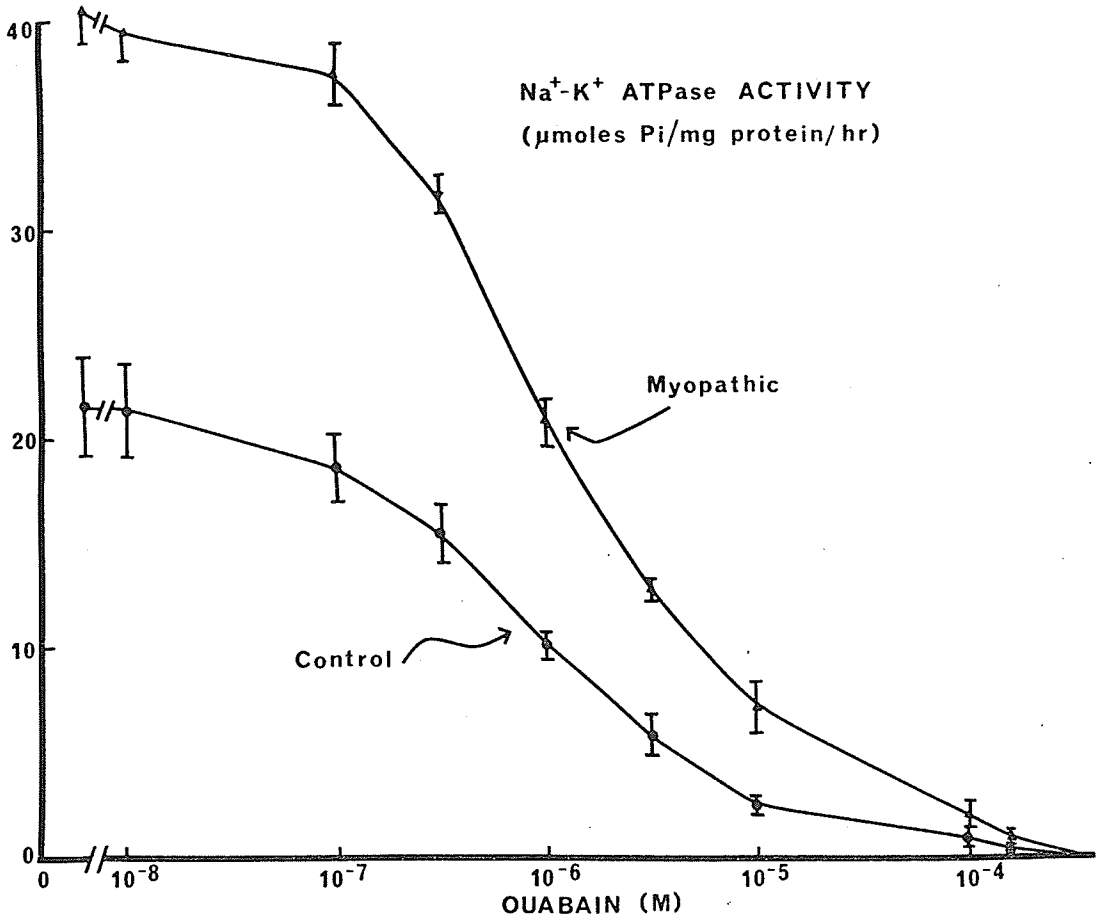

FIGURE 24 Inhibition of $Na^+ - K^+$ ATPase by ouabain. Enzyme preparations isolated from control and failing hearts were incubated in the standard incubation media in the absence or presence of indicated concentrations of ouabain. Results are mean ± S.E. of at least 3 separate experiments with different preparations. It can be observed that ouabain sensitivity of the preparations isolated from control and failing hearts is very similar.

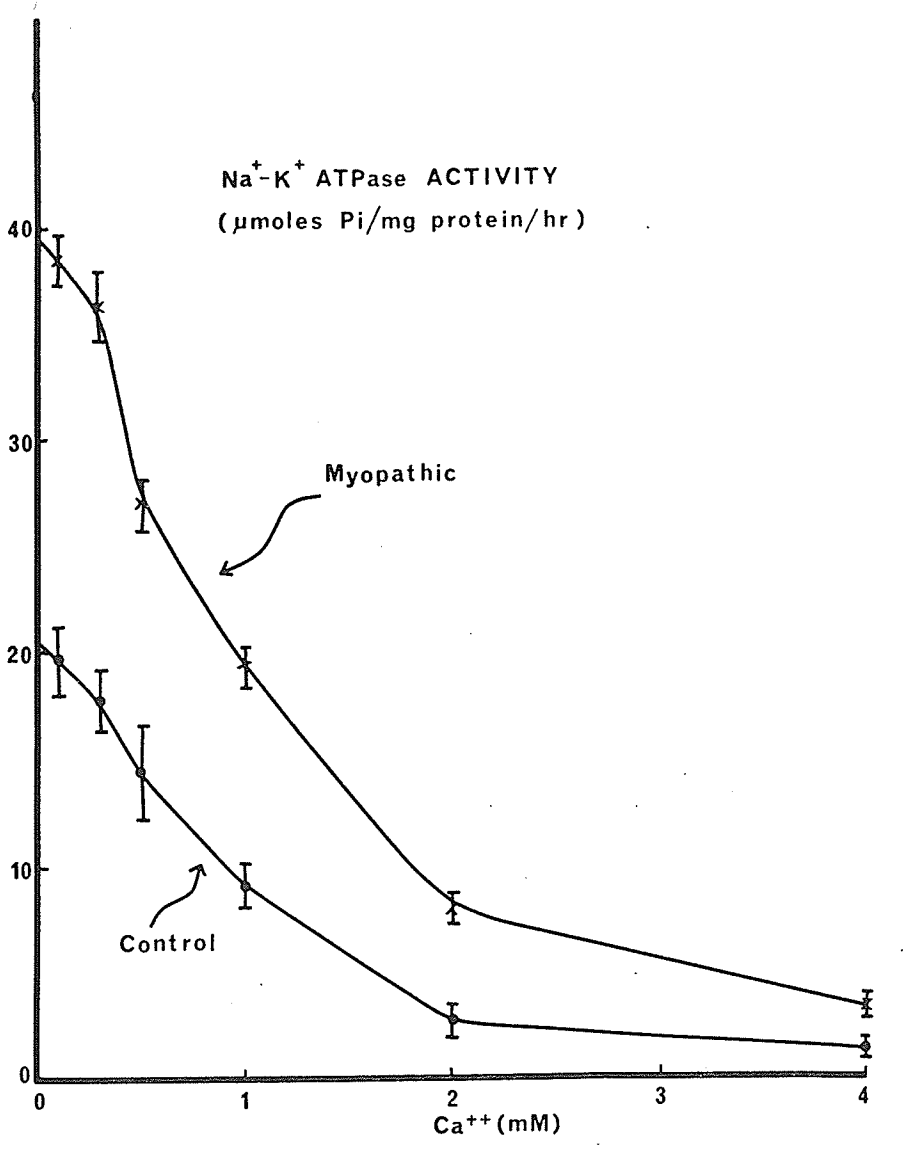

FIGURE 25

Inhibition of $Na^+ - K^+$ ATPase by calcium. The enzyme preparations incubated in the standard media in the absence or presence of indicated concentrations of calcium. The results are obtained from 3 separate preparations. Calcium sensitivity of enzyme preparations isolated from control and failing hearts is very similar.

in this study. The values for enzyme activities in heart homogenates prepared either in 50 mM Tris-HCl, pH 7.6 (Method "A") or 250 mM sucrose containing 10 mM Tris-HCl, pH 7.6 (Method "B") were essentially similar. These values for adenyl cyclase activity are comparable to those reported for other species (70 - 74, 77, 119).

The adenyl cyclase activities of both control and failing heart homogenates were dependent upon Mg^{++} and increased in the presence of NaF and norepinephrine, two well known activators of this enzyme system (75). In one series of experiments, adenyl cyclase activities in the absence and presence of NaF or norepinephrine were determined in the heart homogenates of 210 - 220 day old control and myopathic hamsters (male) and the results are shown in Tables X and XI. No appreciable differences between the control and myopathic hearts with respect to basal or NaF and norepinephrine stimulated adenyl cyclase activities were observed when the estimations were carried out in homogenates prepared according to Method "A". However, a slight decrease in adenyl cyclase activity in the presence of NaF was apparent in myopathic heart homogenates prepared according to Method "B".

In another strain of myopathic hamsters (BIO 82.62), which is also known to develop congestive heart failure (105), adenyl cyclase activities in the heart homogenates were determined in the absence and presence of NaF or norepinephrine. Although no significant differences in the basal or NaF stimulated adenyl cyclase activities were noted between control and myopathic hearts, a slight reduction ($P < 0.05$) in norepinephrine stimulated adenyl cyclase activity was apparent in the failing hearts (Table XII).

TABLE X

Adenyl Cyclase Activities in the Absence or Presence of Various Concentrations of NaF in the Homogenate of Hearts of Control and Myopathic Male Hamsters (210 - 230 day old)

Concentration of NaF (mM)	Adenyl Cyclase Activity			
	Method A		Method B	
	Control	Myopathic	Control	Myopathic
	pmoles cyclic AMP/mg protein/5 min			
-	175 ± 5	173 ± 4	156 ± 5	167 ± 5
2	692 ± 27	569 ± 15	698 ± 27	570 ± 20
5	982 ± 41	817 ± 20	915 ± 34	765 ± 55
10	875 ± 19	781 ± 23	887 ± 33	694 ± 19
20	815 ± 11	730 ± 44	835 ± 29	630 ± 19

Hearts from control and myopathic hamsters were homogenized either in 50 mM Tris-HCl, pH 7.6 (Method A) or in 250 mM sucrose - 10 mM Tris-HCl; pH 7.6 (Method B) and the adenyl cyclase activities of the homogenates were assayed immediately in the medium containing 25 - 30 mM Tris-HCl, pH 7.4 - 7.6, 10 mM KCl, 10 µg pyruvate kinase, 20 mM phosphoenol pyruvate, 2 mM $MgCl_2$, 2 mM ATP-8-C^{14}, in the absence or presence of different concentrations of sodium fluoride in a total volume of 0.1 ml for 5 min at 37°C. The cyclic AMP formed was determined as described in "Methods". Please note that control and failing heart homogenates showed very similar basal as well as fluoride stimulated adenyl cyclase activities. Each value is a mean ± S.E. of 6 experiments.

TABLE XI

Adenyl Cyclase Activities in the Absence or Presence of Various Concentrations of Norepinephrine in the Homogenates of Hearts of Control and Myopathic Male Hamsters (210 - 230 day old)

Concentrations of Norepinephrine (M)	Adenyl Cyclase Activity			
	Method A		Method B	
	Control	Myopathic	Control	Myopathic
	pmoles cyclic AMP/mg protein/5 min			
-	181 ± 7	172 ± 5	156 ± 5	167 ± 5
1×10^{-7}	196 ± 5	195 ± 4	175 ± 5	181 ± 4
1×10^{-6}	247 ± 5	236 ± 7	209 ± 7	220 ± 6
1×10^{-5}	297 ± 4	282 ± 6	275 ± 11	256 ± 4
5×10^{-5}	318 ± 8	299 ± 4	303 ± 9	286 ± 7
1×10^{-4}	327 ± 10	308 ± 7	306 ± 8	297 ± 8

The control and failing hearts were homogenized in 50 mM Tris-HCl, pH 7.4 - 7.6 (Method A) or in 250 mM sucrose - 10 mM Tris-HCl, pH 7.4 - 7.6 (Method B) and assayed as described in Table XI in the presence or absence of varying concentrations of norepinephrine bitartarate for 5 min at 37°C. The cyclic AMP formed was estimated by the paper chromatographic method as described in the "Method" section. Each value is a mean ± S.E. of 6 experiments. Please note there is no difference between the control and failing heart homogenate adenyl cyclase activities in the absence or presence of norepinephrine.

TABLE XII

Adenyl Cyclase Activity of the Heart Homogenate of the Control and BIO 82.62 Myopathic Male Hamsters (210 - 230 day old)

Conditions	Adenyl Cyclase Activity	
	Control	Myopathic (82.62)
	pmoles cyclic AMP/mg protein/ 5 min	
-	156 ± 5	153 ± 6
NaF (8 mM)	830 ± 30	716 ± 44
Norepinephrine (10^{-4}M)	297 ± 8	263 ± 10

Adenyl cyclase activities of heart homogenates of myopathic (BIO 82.62) and control hamsters were determined essentially as described in the "Method" section. Each value is a mean ± S.E. of 6 experiments for control and of 4 experiments for myopathic hearts. Please note that basal, sodium fluoride stimulated and norepinephrine stimulated activities of control are not different than that of myopathic ($P > 0.05$).

The adenyl cyclase activities of the washed cell particles from the control and both strains of myopathic hamster hearts were determined in the absence and presence of norepinephrine or NaF. The enzyme activities in both strains of myopathic hamster hearts washed cell particles were not different ($P > 0.05$) from the control values (Table XIII). In another series of experiments, the heavy microsomal fractions of the control and myopathic (BIO 14.6) 210 - 230 day old hamster (male) hearts were isolated and the adenyl cyclase activities were estimated. The results shown in Table XIV indicate no significant changes ($P > 0.05$) in the basal or NaF stimulated adenyl cyclase activities, but the enzyme activity in the presence of norepinephrine was significantly reduced ($P < 0.05$) in the microsomal fraction of the myopathic hamster hearts.

The adenyl cyclase activities of the hearts from myopathic hamsters at advanced stages of heart failure were also determined. The basal adenyl cyclase activity of the 260 - 275 day old male myopathic hamster heart homogenate was not different ($P > 0.05$) from that of the control (Tables XV and XVI). However, from these tables, it can be seen that the adenyl cyclase activities of the heart homogenates in the presence of various concentrations of NaF was markedly decreased ($P < 0.01$) whereas norepinephrine activation was not observed in the failing heart homogenates. The effects of NaF and norepinephrine were also studied on the washed cell particles obtained from these animals. Fig. 26 indicates a marked reduction in NaF activation of adenyl cyclase activity of the failing heart washed cell particles whereas the responses to norepinephrine were absent (Fig. 27).

In another series of experiments, adenyl cyclase activities were determined in female myopathic hamsters at advanced stages of heart failure. No

TABLE XIII

Adenyl Cyclase Activity of the Heart Washed Cell Particles of the Control and Myopathic Male Hamsters (210 - 230 day old)

Conditions	Adenyl Cyclase Activity		
	Control	Myopathic (BIO 14.6)	Myopathic (BIO 82.62)
A. Norepinephrine (M)	pmoles cyclic AMP/mg protein/5 min		
-	162 ± 7	173 ± 6	162 ± 8
1×10^{-7}	175 ± 5	181 ± 5	172 ± 3
1×10^{-6}	191 ± 5	197 ± 4	188 ± 5
1×10^{-5}	219 ± 5	225 ± 5	232 ± 10
5×10^{-5}	247 ± 6	238 ± 11	250 ± 11
1×10^{-4}	240 ± 6	237 ± 12	236 ± 6
B. NaF (mM)			
-	165 ± 6	171 ± 5	164 ± 7
2	550 ± 35	498 ± 29	580 ± 30
5	818 ± 24	726 ± 13	805 ± 27
10	761 ± 23	691 ± 11	765 ± 26
20	635 ± 16	615 ± 8	710 ± 40

Washed cell particles of control and myopathic (BIO 14.6 and BIO 82.62) are isolated according to that described in the "Methods" section. The particles were incubated at 37°C for 5 min at a protein concentration of 80 - 120 µg. The activities of control and of both strains of myopathic hamsters in the absence or presence of different concentrations of sodium fluoride and norepinephrine were not different from each other.

TABLE XIV

Adenyl Cyclase Activity of the Heart Heavy Microsomal Fraction of the Control and Myopathic Male Hamster (210 - 230 day old)

Condition	Adenyl Cyclase Activity	
	Control	Myopathic
	pmoles cyclic AMP/mg protein/5 min	
Basal	226 ± 12	236 ± 11
NaF (5 mM)	509 ± 20	487 ± 22
Norepinephrine (5×10^{-5}M)	366 ± 15	228 ± 12

Heavy microsomal fractions (10,000 - 40,000 x g) of control and myopathic heart homogenates were incubated in the standard incubation mixture for 5 min at 37°C. The microsomal fractions as judged by enzyme marker activities and electron microscope appearance revealed negligible contaminations. Please note that norepinephrine activation of adenyl cyclase of myopathic hearts was not apparent although basal and sodium fluoride stimulated activities of control and failing heart vesicles are not different from each other. Each value is a mean ± S.E. of 4 experiments.

TABLE XV

Adenyl Cyclase Activities in the Absence or Presence of Various Concentrations of NaF in the Homogenates of Hearts of Control and Myopathic Male Hamsters (260 - 275 day old)

Concentration of NaF (mM)	Adenyl Cyclase Activity			
	Method A		Method B	
	Control	Myopathic	Control	Myopathic
	pmoles cyclic AMP/mg protein/ 5 min			
-	153 ± 5	155 ± 4	144 ± 9	153 ± 8
2	506 ± 15	381 ± 9	588 ± 25	393 ± 14
5	935 ± 23	421 ± 11	889 ± 17	431 ± 12
10	833 ± 25	441 ± 15	823 ± 14	429 ± 14
20	755 ± 16	396 ± 7	856 ± 14	398 ± 6

Adenyl cyclase activities of heart homogenates of control and myopathic hamsters at advanced stages of heart failure were determined as described in Table X. At this time, the fluoride stimulated activities of failing hearts were markedly reduced in comparison to the control ($P < 0.05$), although basal activities were not different ($P > 0.05$). Each value is a mean ± S.E. of 6 experiments.

TABLE XVI

Adenyl Cyclase Activities in the Absence or Presence of Various Concentrations of Norepinephrine in the Homogenates of Hearts of Control and Myopathic Male Hamsters (260 - 275 day old)

Concentration of Norepinephrine (M)	Adenyl Cyclase Activity			
	Method A		Method B	
	Control	Myopathic	Control	Myopathic
	pmoles cyclic AMP/mg protein/5 min			
-	153 ± 5	155 ± 4	144 ± 9	153 ± 8
1×10^{-7}	173 ± 6	158 ± 4	158 ± 8	163 ± 4
1×10^{-6}	191 ± 5	160 ± 4	182 ± 6	163 ± 6
1×10^{-5}	234 ± 5	159 ± 5	218 ± 8	161 ± 9
5×10^{-5}	256 ± 5	158 ± 5	296 ± 5	160 ± 10
1×10^{-4}	265 ± 6	163 ± 5	271 ± 3	170 ± 9

Adenyl cyclase activities of heart homogenates of control and myopathic hamsters were assayed in the absence or presence of different concentrations of norepinephrine. It can be seen that norepinephrine activates the adenyl cyclase of the control heart homogenates while such response to norepinephrine was not apparent with failing heart homogenates ($P < 0.05$). Each value is a mean ± S.E. of 6 experiments.

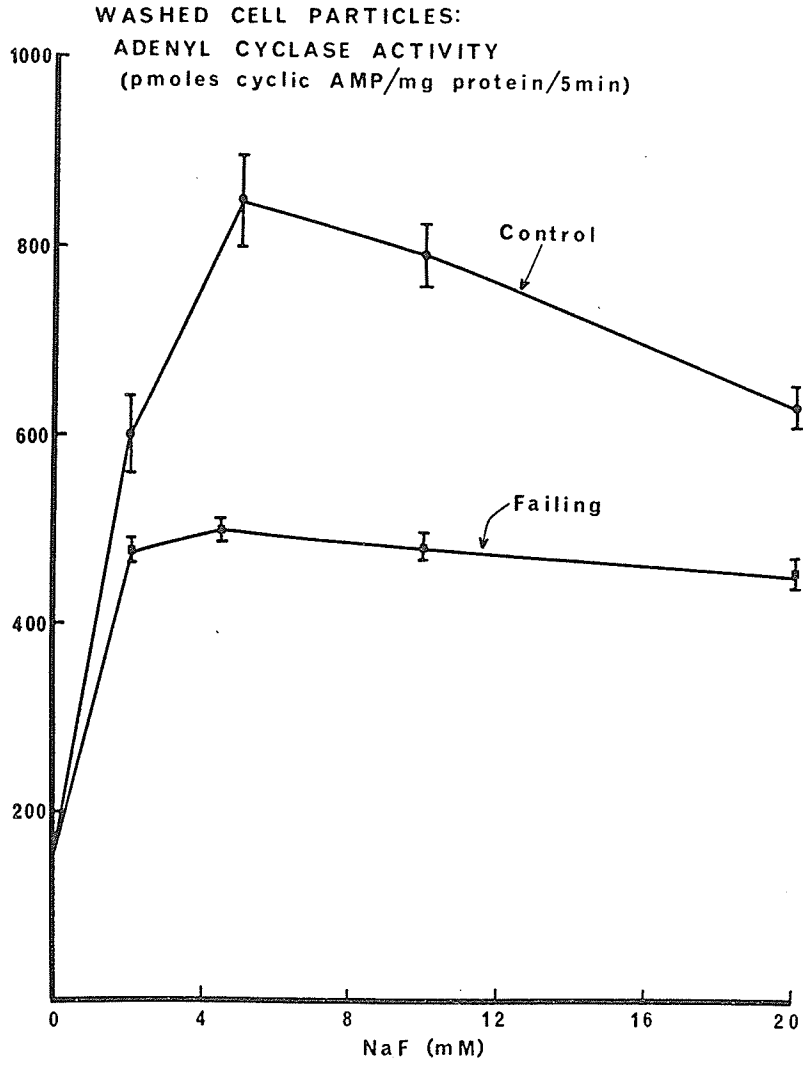

FIGURE 26

Response to NaF. Washed cell particles were isolated from control and failing hearts as described in "Methods". The particles were incubated in the absence or presence of different concentrations of NaF for 5 minutes at 37°C in the standard assay media ("Methods"). Please note that NaF stimulation of adenyl cyclase of the particles isolated from failing hearts is markedly reduced in comparison to control ($P < 0.05$). Each value is a mean $\pm$ S.E. of 6 experiments.

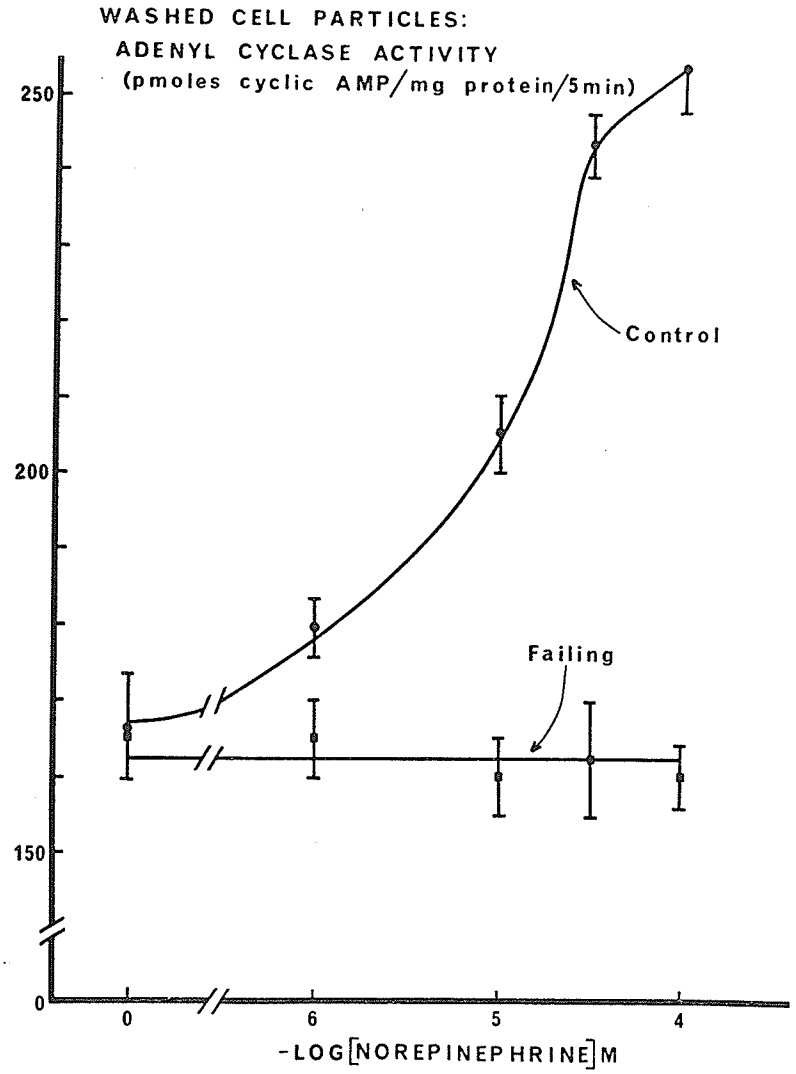

FIGURE 27

Response to norepinephrine. Washed cell particles were incubated in the absence or presence of indicated concentrations of norepinephrine. Please note that norepinephrine activates adenyl cyclase of control heart particles ($P < 0.01$) while such activation is not apparent with failing heart particles ($P < 0.05$). Results are obtained from 6 separate preparations.

change in the basal activity of enzyme in heart homogenates was observed in these groups of animals (Tables XVII and XVIII). However, the responses to NaF were markedly decreased ($P < 0.01$) whereas norepinephrine activation was not apparent in the heart homogenates of the 210 - 230 day old female myopathic hamsters with severe heart failure.

The phosphodiesterase enzyme activities of the heart homogenates from the control and myopathic hamsters were also determined and the results are shown in Table XIX. The enzyme activities in various groups of myopathic hamster hearts were not different ($P > 0.05$) from their respective control values.

TABLE XVII

Adenyl Cyclase Activities in the Absence or Presence of Various Concentrations of NaF in the Homogenates of Hearts of Control and Myopathic Female Hamsters (210 - 230 day old)

Concentration of NaF (mM)	Adenyl Cyclase Activity			
	Method A		Method B	
	Control	Myopathic	Control	Myopathic
	pmoles cyclic AMP/mg protein/5 min			
-	158 ± 6	168 ± 6	167 ± 6	174 ± 7
2	570 ± 34	390 ± 10	667 ± 25	473 ± 14
5	796 ± 26	434 ± 8	895 ± 30	506 ± 9
10	782 ± 19	403 ± 6	882 ± 26	494 ± 5
20	723 ± 18	406 ± 7	798 ± 25	427 ± 15

Heart homogenates prepared in 50 mM Tris-HCl, pH 7.6 (Method A) or 250 mM sucrose - 10 mM Tris-HCl, pH 7.6 (Method B) as described in "Methods". Adenyl cyclase activities of homogenates assayed in the standard assay mixture. Each value is a mean ± S.E. of 6 experiments. It can be seen that NaF stimulated activities of failing heart homogenates are markedly lower than that of the control hearts ($P < 0.05$).

TABLE XVIII

Adenyl Cyclase Activities in the Absence or Presence of Various Concentrations of Norepinephrine in the Homogenates of Hearts of Control and Myopathic Female Hamsters (210 - 230 day old)

Concentration of Norepinephrine (M)	Adenyl Cyclase Activity			
	Method A		Method B	
	Control	Myopathic	Control	Myopathic
	pmoles cyclic AMP/mg protein/5 min			
-	158 ± 6	168 ± 5	167 ± 6	174 ± 7
1×10^{-7}	173 ± 6	169 ± 6	176 ± 6	179 ± 6
1×10^{-6}	185 ± 6	174 ± 8	193 ± 5	182 ± 7
1×10^{-5}	217 ± 5	171 ± 7	224 ± 5	177 ± 7
5×10^{-5}	249 ± 5	188 ± 8	259 ± 6	177 ± 8
1×10^{-4}	256 ± 5	178 ± 4	258 ± 4	175 ± 5

Norepinephrine stimulated adenyl cyclase activities of control and failing heart homogenates were determined at different norepinephrine concentrations in the standard assay mixture. Please note that norepinephrine activates the enzyme of control heart homogenate but not of failing heart homogenates ($P < 0.05$), $n = 6$.

TABLE XIX

Phosphodiesterase Activities of Heart Homogenates

		mμmoles cyclic AMP/mg Protein	
	Age & Sex	Method A	Method B
Control	230 days, Female	5.1 ± 0.4	4.9 ± 0.2
Myopathic		5.3 ± 0.3	5.0 ± 0.4
Control	210 - 230 days, Male	6.2 ± 0.5	5.8 ± 0.4
Myopathic		6.5 ± 0.3	6.1 ± 0.5
Control	260 - 275 days, Male	5.5 ± 0.4	5.9 ± 0.5
Myopathic		5.7 ± 0.3	6.0 ± 0.4

Phosphodiesterase activities of heart homogenates were measured as described in "Methods". Time of incubation was 15 min at 37°C. Please note that activities of control and failing hearts are not different from each other ($P > 0.05$). Each value is a mean ± S.E. of at least 5 experiments.

DISCUSSION

1. Defect in Calcium Transport by Subcellular Particles of Failing Heart

On the basis of their ability to accumulate calcium, heavy microsomes mainly and mitochondria to a certain extent are considered to regulate the concentration of free calcium in the heart (56 - 63). If the control of intracellular calcium is important in heart function, a change in the capacity of various subcellular fractions to accumulate calcium may contribute to the pathogenesis of heart failure. The results of the present study reveal that calcium binding by both mitochondria and heavy microsomes of the failing heart from the myopathic hamster was markedly decreased in comparison to the control. This observation suggests the association of abnormal calcium pump mechanism with heart failure in these hamsters but does not in any way establish the cause-effect relation between molecular and functional events. Particularly, in the hearts of diseased hamsters of the same age group a marked decrease in creatine phosphate, ATP, ATP/ADP ratio and ATP/AMP ratio has been observed (103) and this suggests an abnormality in the high energy phosphate stores. Therefore, unless an extensive study concerning the changes in the process of energy generation and utilization as well as in calcium accumulating abilities of the subcellular fractions during the course of development of congestive heart failure is complete, the primary biochemical lesion responsible for heart failure in this disease model remains a matter of speculation.

Although ATP-dependent calcium binding by both the subcellular fractions declined markedly in the failing heart of the myopathic hamster, no change in ATPase activity of mitochondria or heavy microsomes was noted in this

study. Thus the observed decrease in calcium binding by subcellular fractions can not be explained on the basis of changes in ATP hydrolysis. It is also unlikely that this observation can be due to the presence of inert protein contaminant in the pellet obtained from the failing heart, since both the fractions showed similar yield from the control and diseased hearts. The determination of activities of marker enzymes in these subcellular fractions also did not support this contention. It is further substantiated by our experiments in which oligomycin and sodium azide were employed during the study of calcium binding by the subcellular fractions. If the sensitivity of subcellular particles to homogenization is greater in failing heart than in the control heart, a greater loss of calcium binding may result during isolation. This possibility is unlikely since homogenization of the failing heart for 20 seconds yielded fractions having the same calcium binding capacity as those obtained after 40 seconds of homogenization.

We have shown in this study that calcium uptake (in the presence of oxalate) by cardiac heavy microsomes of myopathic hamsters with moderate degree of heart failure was not decreased significantly while calcium binding (in the absence of oxalate) was markedly reduced. This may mean that either the process of calcium uptake is less sensitive than that of calcium binding or this measure of calcium transport (calcium uptake) is influenced at a later stage of heart failure. A marked reduction in calcium uptake by the heavy microsomal fraction obtained from 260 - 275 day old myopathic hamsters with advanced degree of heart failure was observed in this study. It may be noted that Gertz et al (104) have reported a reduction of ATP-dependent calcium oxalate pumping by the cardiac heavy microsomes by 22% in myopathic hamsters of 200 days age and by 30 to 77%

in animals of 300 days age. It has also been shown that in the failing rat heart perfused with substrate-free medium, calcium binding by subcellular fractions decreased at the onset of failure whereas the defect in calcium uptake was delayed and was associated with late stages of failure (68). Some investigators (57) have suggested that calcium binding by the subcellular fraction is a more physiological measure of calcium transport than calcium uptake in the presence of oxalate. It has been suggested that changes in calcium uptake in the presence of oxalate, which occur in the final stage of heart failure, probably represent irreversible disorganization of the intracellular membranes (68). It is likely that delayed changes in calcium uptake by the heavy microsomes are associated with irreversible state of heart failure in myopathic hamsters.

It was interesting to observe that in myopathic hamsters with a moderate degree of heart failure, calcium accumulation by cardiac mitochondria in the absence or presence of Pi and succinate was decreased. This suggests the possibility of a greater degree of change in the functional integrity of the mitochondrial membranes than in the microsomal particles. Defect in oxidative phosphorylation by the mitochondria from the hearts of myopathic hamsters of the same age group as employed in this study has also been reported by other workers (98 - 101) and a decrease in calcium uptake by these mitochondria has been indicated (100, 101). Thus it appears that "calcium pump" mechanism in mitochondria particularly and in heavy microsomes to a certain extent are defective in the early (initial) stages of heart failure in the myocardium of the myopathic hamsters. At present it is difficult to rule out whether a greater degree of damage to mitochondria is a result of the isolation procedures employed in this study or is a consequence of the

in vivo events leading to alteration in the integrity of these subcellular particles. Such a damage could be due to an increase in lysosomal activity in the myopathic hearts (133).

Under a variety of experimental conditions, we have clearly demonstrated a decrease in calcium transport by subcellular fractions of the failing heart. The reduction in calcium transport by mitochondrial or microsomal particles has been shown to be due to a decrease in the velocity of reaction (V_{max}) rather than any alterations in the affinity of calcium for its binding sites. It should, however, be noted that there was an increase in the affinity constants of high affinity binding sites for ATP and of low affinity binding sites for Mg^{++} in the heavy microsomal membranes of the myopathic hearts. It is also likely that the number of sites participating in calcium transport by these particles are decreased in failing heart. Alternatively, such a defect in calcium transport is due to some conformational changes in the membranes, which are likely to occur as a result of altered chemical composition of these subcellular particles in failing hearts. Thus a detailed analysis of phospholipid and protein composition of the subcellular membranes of the myopathic hearts is required for a plausible explanation.

2. Abnormality in $Na^+ - K^+$ ATPase of Failing Heart

It was interesting to find that the activity of $Na^+ - K^+$ stimulated ATPase in the hearts of myopathic hamsters with a moderate degree of failure was higher than that of the control. This change is not likely to be due to different degrees of purification of the enzymes since both the control and myopathic preparations were made concomitantly under similar conditions. Furthermore, higher activity of $Na^+ - K^+$ ATPase in the failing hearts was also apparent in both

fractions obtained after treatments with deoxycholate and NaI during the process of isolation. In contrast to the observations reported here, $Na^+ - K^+$ ATPase activity of the failing hearts of guinea pig has been reported to be reduced (81). This may be due to either differences in experimental models, the degree of failure of the hearts employed for enzyme determination or the methods of isolation. We have not attempted to measure the enzyme activities in the hearts of myopathic hamsters with advanced stages of heart failure. Other investigators (81) however, have not described their experimental conditions in a brief report. Therefore, it is difficult to resolve the discrepancy in results at present. It should, however, be noted that $Na^+ - K^+$ ATPase activity of the skeletal muscle of the myopathic hamsters was also found to be higher than the normal (134).

The intracellular level of Na^+ has been observed to be higher and that of K^+ to be lower in the myopathic hearts than those in the normal myocardium (87, 135). Since $Na^+ - K^+$ ATPase is believed to be involved in the transport of these ions across cell membrane (82, 83), the increased specific activity of $Na^+ - K^+$ stimulated ATPase in the myopathic heart may be considered to serve as a compensatory mechanism, and in doing so may lower the high energy phosphate stores in the myocardium as seen in this model of heart failure (103). Thus, it is tempting to speculate that increased activity of $Na^+ - K^+$ ATPase in myopathic hearts may be one of the important factors which may lead to deteriorating the myocardial function by lowering the concentration of ATP which is essential for the contractile process (136).

Although $Na^+ - K^+$ ATPase activity of the myopathic hearts was higher than the normal, no difference in its sensitivity to ouabain or calcium the two well

known inhibitors of this enzyme (82, 83) was noted. Likewise, no changes were seen in the affinity for substrate (K_m) or in the concentrations of Na^+ and K^+ for half maximal stimulation of the enzyme. From the sigmoidal shape of the curves for substrate velocity reactions under different experimental conditions, the $Na^+ - K^+$ ATPase obtained from the myopathic hamsters exhibited an allosteric nature of activation kinetics similar to that noted for the enzyme from control hearts. This is further supported by the fact that n values obtained from Hill plots of the data were > 1 and were not different for the control and myopathic hearts. These results indicate cooperativity of interaction between these activating ions as well as of its substrate. The allosteric nature of $Na^+ - K^+$ ATPase isolated from hamster hearts is in agreement with our earlier observations for dog heart enzyme (137). In spite of the fact that $Na^+ - K^+$ ATPase activity was higher in fractions obtained from the myopathic hearts under different experimental conditions such as various concentrations of Na^+, K^+, H^+, and Mg^{++} ATP, it is pointed out that the interpretation of these results obtained in vitro experiments be extrapolated to in vivo situation with a great deal of caution.

The reasons for the increased specific activity of $Na^+ - K^+$ ATPase in myopathic heart are not clear at present; however, an increase in V_{max} for this enzyme in failing hearts was noted in comparison with control. Further experiments are clearly needed to understand this problem in greater detail. The most fruitful area of investigation in this regard would be to study the chemical composition of these fractions containing $Na^+ - K^+$ ATPase activity. Since certain types of phospholipids have been reported to be important for the activity of $Na^+ - K^+$ ATPase (138), it is likely that the observed changes in the enzyme activity in

myopathic hearts may be due to changes in lipid composition of these membranes. At any rate, the alteration in $Na^+ - K^+$ ATPase activity in myopathic heart further supports our contention concerning an abnormality of cardiac membranes in heart failure.

3. Alteration in Adenyl Cyclase of Failing Heart

In this study we have observed that adenyl cyclase activities of the heart homogenates and washed cell particles in the absence and presence of NaF or norepinephrine did not change appreciably in the myopathic hamsters (BIO 14.6) with a moderate degree of heart failure. Likewise these enzyme activities in another strain of myopathic hamsters (BIO 82.62) with a moderate degree of heart failure were not altered significantly. On the other hand, adenyl cyclase activities of the heart homogenates or washed cell particles in the presence of NaF were decreased in both male and female myopathic hamsters (BIO 14.6) with an advanced degree of heart failure. Although the basal adenyl cyclase activity in the heart homogenates and washed cell particles of animals with severe heart failure did not change, the activation of this enzyme by various concentrations of norepinephrine was not apparent. This clearly indicates dramatic changes in the myocardial adenyl cyclase system in myopathic hamsters at late stages of heart failure. Although some degree of alteration in adenyl cyclase system of the heavy microsomal fraction of hearts with moderate degree of failure was noted, the significance of this observation is not clear at present.

Conflicting reports concerning changes in adenyl cyclase activity in heart failure have appeared in the literature. On one hand, Sobel et al (69) have reported depressed adenyl cyclase activity in failing guinea pig hearts while

on the other hand Gertler et al (73) failed to observe such a change. Although Gold et al (70) did not observe any alteration in myocardial adenyl cyclase activity in the absence or presence of fluoride and norepinephrine in chronic heart failure induced in cats by occluding the pulmonary artery, glucagon was found unable to activate this enzyme. Recently, we have observed a marked reduction in the responses of myocardial adenyl cyclase to epinephrine and fluoride in hearts which failed to generate contractility due to substrate-lack; however, no alteration in enzyme was observed in hearts which failed to generate contractile force by about 50% of the control value (74). In the present study no appreciable changes in adenyl cyclase were seen in myopathic heart with a moderate degree of heart failure whereas its responses to norepinephrine and fluoride were markedly altered in late stages of failure. The discrepancy in results from various laboratories may be due to either the difference in the type of heart failure or the degree of heart failure.

There is considerable evidence that cyclic AMP plays a unique role in myocardial metabolism (75, 78, 79) and is considered to mediate metabolic responses due to sympathetic impulses to the heart (139). Myocardial cyclic AMP concentration depends upon the activity of at least two enzymes: adenyl cyclase, regulating synthesis and phosphodiesterase, regulating degradation of the metabolite (75). Since adenyl cyclase activation in the presence of NaF was diminished whereas phosphodiesterase activity was unchanged in hearts of animals with severe degree of failure, it is conceivable that these hearts are unable to maintain an adequate level of intracellular cyclic AMP. The inability of norepinephrine to activate adenyl cyclase in hearts at late stages of failure may suggest a defect in

the mediation of the metabolic effects of sympathetic system.

Some investigators are of the opinion that adenyl cyclase - cyclic AMP system is involved in positive inotropic action of catecholamines (72, 140 - 142). The observed loss of adenyl cyclase activation by norepinephrine, a well known adrenergic neurotransmitter, would tend to favour the view that further impairment of myocardial performance of myopathic hearts with an advanced degree of failure could be due to a loss of adrenergic support. However, such a suggestion should be considered with due caution since the evidence concerning the involvement of cyclic AMP in myocardial contractility is not established unequivocally (143 - 145). Furthermore, changes in myocardial contractility have been shown to be independent of cyclic AMP (146, 147). We have recently provided (115) evidence against the involvement of cyclic AMP in cardiac contraction through its effect on the sarcotubular system (148). Henry and Sobel (149) have also suggested that the impaired performance of the failing heart does not depend on diminished accumulation of cyclic AMP under basal conditions or following adrenergic stimulation. Until conclusive evidence concerning the direct involvement of cyclic AMP in the contractile process is available, we consider that impaired performance of myopathic hearts be explained on the basis of insufficient availability of ATP (103) and a defect in "calcium pump" mechanism in this model of heart failure.

Our inability to observe changes in the basal as well as fluoride and norepinephrine stimulated adenyl cyclase activities in heart homogenates or washed cell particles of myopathic hamsters with a moderate degree of heart failure suggest that alterations in the enzyme activity in the presence of its

activators at late stages of heart failure are secondary to the impairment of cardiac function. In view of the fact that adenyl cyclase is a membrane-bound enzyme, it is likely that diminished activity in the presence of various doses of fluoride and a marked loss of norepinephrine activation are due to certain changes in the integrity of cardiac cell membrane of myopathic hearts at an advanced degree of failure. This is supported by our finding (77, 150) that treatment of dog heart membranes with phospholipase c or trypsin decreased adenyl cyclase activity in the presence of epinephrine or fluoride.

It was interesting to observe that in hearts with an advanced degree of failure, the activation of adenyl cyclase by NaF was diminished whereas stimulation by norepinephrine was not observed. Likewise, in the heavy microsomal fraction obtained from the hearts at a moderate degree of failure, activation of adenyl cyclase by norepinephrine was decreased whereas that by NaF did not alter significantly. These results can be explained on the basis that there are differences in the sites or mechanisms of adenyl cyclase activation by norepinephrine and fluoride. It has been also reported that adenyl cyclase of solubilized myocardial preparation was activated by NaF but not by catecholamines (151). Certain difference in the mode of activation of heart sarcotubular adenyl cyclase by NaF and catecholamines have been observed in this laboratory (77). These observations lend support to the suggestion that catecholamines interact with the adrenergic receptor of the membrane and thereby activate adenyl cyclase, possibly through conformational changes, whereas fluoride has its binding site in the enzyme molecule itself and thus activates directly (75). Since most of the adenyl cyclase activity in the washed cell particles is considered to be of sarcolemmal origin (77, 119) the

observed changes in this enzyme in failing heart would further support the notion that cardiac membranes are defective in heart failure.

CONCLUSIONS

In this study we have examined the abilities of mitochondrial and heavy microsomal membranes of the control and myopathic hamsters (BIO 14.6) hearts to transport calcium. In addition, the activities of membrane-bound enzymes, $Na^+ - K^+$ ATPase and adenyl cyclase were determined under various experimental conditions. From the data obtained in this study, the following conclusions are drawn:

1. The ability of mitochondria to bind and accumulate calcium in the presence of ATP was impaired in myopathic hamsters with a moderate degree of heart failure.

2. The ability of heavy microsomes to bind calcium in the presence of ATP was decreased in hearts with a moderate degree of failure whereas calcium accumulation in the presence of ^{oxalate} was depressed in hearts with advanced stages of failure.

3. The activity of $Na^+ - K^+$ ATPase of myopathic hearts with a moderate degree of failure was markedly increased in comparison to control.

4. The basal adenyl cyclase activity of the failing myopathic hearts was not different from the control. Although no appreciable changes in the NaF and norepinephrine stimulated activities of adenyl cyclase occured in hearts with a moderate degree of failure, the activation by NaF was markedly diminished and that by norepinephrine was not observed in hearts with advanced stages of failure.

5. On the basis of information obtained in this study it is difficult to identify the primary biochemical lesion responsible for the pathogenesis of heart

failure in myopathic hamsters; however, the observed alterations in the "calcium pump" of mitochondria and heavy microsomes as well as in the activities of Na^+ - K^+ ATPase and adenyl cyclase in the presence of its activators reveal defects in cardiac membrane events associated with heart failure.

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