THE EFFECTS OF VARIOUS INTERVENTIONS ON MYOCARDIAL CATION CONTENTS DURING INDUCTION OF THE "CALCIUM PARADOX" IN ISOLATED PERFUSED RAT HEART

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LAURI E.C. ALTO

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

MASTER OF SCIENCE

to my parents

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ABSTRACT

Reperfusion of Ca^{2+}-deprived hearts with normal calcium containing medium, has been shown to produce dramatic changes in contractile function and ultrastructure of the myocardium. This "calcium paradox" phenomenon has been suggested to be due to an intracellular calcium overload. The present study was undertaken to determine if reperfusion of isolated rat hearts following Ca^{2+}-free exposure, is associated with changes in myocardial Ca^{2+} as well as other cation (Mg^{2+}, K^{+} and Na^{+}) contents.

Reperfusion of the isolated hearts after 5 minutes of Ca^{2+}-free perfusion, was found to produce marked increases in myocardial Ca^{2+} and Na^{+} and decreases in Mg^{2+} and K^{+} contents. The critical concentration of calcium in the perfusing medium, during the period of Ca^{2+}-deprivation, which allowed for the occurrence of reperfusion-induced changes in myocardial cation contents, was found to be less than 0.1mM. In addition, more than 1 minute of Ca^{2+}-free exposure was required in order to induce changes in myocardial cation contents due to reperfusion. The reperfusion-induced alterations in myocardial Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} contents, after 5 or 10 minutes of Ca^{2+}-free perfusion, were found to be time-dependent. The above cation changes were prevented if the Ca^{2+}-free perfusion medium contained low (35mM) Na^{+} or was cooled to room temperature (21°C). These two interventions allowed for the complete recovery of contractile force, upon reperfusion, which otherwise was absent.

Although the loss of recovery of contractile force due to reperfusion of the Ca^{2+}-deprived hearts, was linearly related to increases in myocardial Ca^{2+} and Na^{+}, and decreases in Mg^{2+} and K^{+} contents, the data at hand do not reveal the existence of a cause and effect relationship.

In another series of experiments, the composition of the reperfusion medium was altered in order to investigate the nature of reperfusion-induced changes in the cation contents of hearts perfused for 5 minutes with zero calcium containing media. The alterations in myocardial cation contents were dependent upon the concentration of Ca^{2+} in the reperfusion medium as well as the duration of reperfusion. Decreasing the Na^{+} concentration of the reperfusion medium depressed the increments in myocardial Na^{+} contents over time, while the increments in Ca^{2+} content were enhanced. On the other hand, a decrease in the K^{+} concentration augumented K^{+} loss, but did not alter the increase in myocardial Ca^{2+} content due to reperfusion. Changes in the Mg^{2+} concentration did not affect the reperfusion-induced alterations in myocardial Ca^{2+} content significantly. The extent of changes in myocardial cation contents due to reperfusion, was not significantly affected by the presence of verapamil (.1mg/l), D_{600} (.1mg/l) or propranolol (1 mg/l) in the reperfusion medium.

These results are consistent with the view that the "calcium paradox" is a complex phenomenon and may possibly be associated with enhanced calcium influx during reperfusion, as well as alterations in other myocardial cations.

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INTRODUCTION AND STATEMENT OF THE PROBLEM

Calcium ion is now considered to be the final mediator in the excitation-contraction coupling process of the myocardium. It is obvious then, that the regulation of calcium is critical with respect to the maintenance of normal contractile function. The majority of calcium regulation is believed to be accomplished by four cellular organelles; sarcolemma, mitochondria, sarcoplasmic reticulum and myofibrils. From this it can be appreciated that the major consequence of membrane alteration would be an abnormality in calcium transport, which could be conceived to result in an intracellular calcium deficiency or an intracellular calcium overload; depending upon the type of membrane change for different types of failing heart.

When isolated hearts are perfused with zero Ca^{2+}-containing media, contractile activity ceases. This is most likely due to reduced entry of calcium during the plateau phase of the cardiac action potential and this condition is believed to represent an intracellular calcium deficiency. Prolonged periods of Ca^{2+}-deprivation have been found to produce ultrastructural damage similar to that seen in ischemic myocardium. If hearts, deprived of calcium for short periods, are then reperfused with media containing normal calcium, a whole series of deleterious physiological and biochemical events ensue. Irreversible cardiac contracture and severe ultrastructural derangement are the two most profound al-

terations. These changes are believed to be accompanied by an intracellular calcium overload. This condition has been termed the "calcium paradox". Though these two models are widely employed for studying the effects of intracellular calcium deficiency and overload, there is no adequate information available in the literature on changes in myocardial calcium content.

This study was designed to investigate changes in myocardial cation contents during conditions believed to cause calcium overload and depletion. We propose to answer several specific questions. Is there a calcium overload and deficiency? Is there a critical concentration of calcium required for induction of the "calcium paradox" in isolated rat hearts? What is the time dependency of this phenomenon? Do other myocardial cations participate in the response? Finally, can various interventions in the perfusion media modify the changes in myocardial cation contents which occur during the time-course of the "calcium paradox"?

REVIEW OF LITERATURE

The pathogenesis of heart failure has fascinated the experimental cardiologist from the time the involvement of cardiac muscle in this condition was recognized. Considerable effort has been afforded to the identification of a single causal factor responsible for myocardial failure. Two major theories namely, abnormalities in energy metabolism and defects in calcium metabolism, have been proposed to explain the molecular events leading to the development of heart failure. At first, the focus of attention revolved around studying changes in myocardial metabolism, where it was suggested that a biochemical lesion may be in those processes concerned with ATP generation or ATP utilization (1). Abundant evidence has appeared in the literature to justify this postulate (2,3), and the research in this regard has centered around determining changes in the high energy phosphate stores, mitochondrial oxidative phosphorylation activity and myofibrillar ATPase activity. However, heart failure has been shown to occur without any change in these parameters (4), and this has formed the basis for questioning the validity regarding the role of primary defects in myocardial metabolism in the genesis of heart failure. In view of a great deal of information available on this subject in the form of several reviews (1-3), no effort has been made to discuss this topic in further detail.

The more recent of the two theories of heart failure

involves the condition of impaired calcium regulation (5), resulting in an intracellular calcium deficiency or an intracellular calcium overload. The basis for this view stems from the vital role played by calcium in the excitation-contraction coupling process of the myocardium. In this regard it should be pointed out that Ringer (6) showed the loss of contractile function when the isolated frog heart was perfused with Ca^{2+}-free medium. The importance of calcium was further emphasized by Heilbrunn and Wiercinski (7) who demonstrated that an intracellular injection of calcium was capable of initiating contraction. Later it was found that the ionized form of calcium was the moiety responsible for contractile activity and the force of contraction was directly related to the amount of ionized calcium in the cell (8,9). Research to date has established the fact that calcium is the final mediator in the excitation-contraction coupling process of the heart (10-13). In view of the latter it is apparent that the regulation of calcium in the heart is of utmost importance in the maintenance of normal contractile function.

According to the current state of the excitation-contraction coupling hypothesis, four cellular organelles namely, sarcolemma, mitochondria, sarcoplasmic reticulum and the contractile proteins, are believed to be responsible for the majority of calcium regulation in the heart. Two recent reviews dealing with the regulatory role of membrane systems in normal and diseased heart function, have appeared in the

literature (14,15). It has been suggested (5) that the major consequence of membrane alterations is an abnormality of calcium transport which could produce either an intracellular calcium deficiency or an intracellular calcium overload, depending on the type of membrane change at different stages of heart failure. Both intracellular calcium deficiency and intracellular calcium overload have been shown to produce functional, metabolic and structural derangements in the myocardium under *in vitro* experimental conditions (16-21).

Intracellular calcium deficiency has been found to cause myocardial cell damage similar to that seen in certain types of heart failure (5,19,22,23), while intracellular calcium overload is believed to be a prominent factor in the development of myocardial lesions (24,25). Perfusion of the isolated rat heart with calcium-free medium is considered to produce an intracellular calcium deficiency (26), whereas reperfusion of calcium-depleted hearts is thought to produce an intracellular calcium overload (26). This experimental protocol has been utilized for diverse studies (17-20,27). Its *in vivo* relevance however, has always been treated with some caution when describing heart disease and failure. Since its first demonstration in 1883 (6), contractile failure of the isolated heart due to Ca^{2+}-free perfusion, has been shown to occur by various investigators (17,18,27,28). The onset and eventual cessation of mechanical activity occurs within the first 30 seconds of Ca^{2+}-free perfusion of isolated rat hearts. This is in contrast to the slower loss of contractile

force in cat and rabbit preparations (29-32). This loss of contractile function has been shown to occur in the absence of any alteration in the surface electrical activity of the heart (20,28,33,34). On the basis of ion fluxes (11,31) the loss in contractility can be conceived to be due to decreased calcium entry during excitation of the myocardium. The dissociation of electrical and mechanical events has also been suggested to be due to the removal of a labile calcium component within the first 30 seconds of Ca^{2+}-free perfusion (16). A decline in tissue or mitochondrial calcium of approximately 32% of total calcium, appeared to represent the compartment associated with the decrease in contractile force. During the loss of this calcium no structural alterations were apparent. A second more stable compartment, representing 20% of myocardial calcium, was depleted only after 10 minutes of Ca^{2+}-free perfusion. At this point marked ultrastructural damage was observed. These 'time of Ca^{2+}-free-perfusion' dependent alterations in cardiac ultrastructure have been observed by other investigators (16,19-21,35). The absolute times tend to vary, probably due to slight methodological differences, but the concensus of opinion is that ultrastructure is unchanged during 4-5 minutes of Ca^{2+}-free perfusion, while longer exposure to calcium-free media results in sarcolemmal disruption, ultrastructural changes in mitochondria and sarcoplasmic reticulum, separation of gap junctions and intercalated discs and transformation of the myofibrils from cylinders to globules. Similar structural

changes have been observed in ischemic human and canine myocardia (23,36). These data are not taken to mean that brief periods of Ca^{2+}-free perfusion are without effect on the myocardium. While some investigators have found no change (33,34), others have pointed to enhanced sarcolemmal permeability to calcium after a short period of Ca^{2+}-free perfusion (27,37). It is possible that Ca^{2+} is removed from the membrane itself during Ca^{2+}-free perfusion, which may render intramembranous proteins more labile (38) and so alter membrane function with respect to calcium movements, permeability in general and various enzymatic activities. Calcium lack has been shown to change the arrangement of cardiolipin molecules in membranes (37). This is consistent with the view that calcium plays an essential role in membrane integrity (37,39,40).

Perfusion of the heart with Ca^{2+}-free medium has been shown to produce dramatic changes in different membrane functions. Sarcolemmal enzyme alterations have been observed during Ca^{2+}-free perfusion. After 20 minutes Ca^{2+}-free perfusion of the isolated rat heart, adenylate cyclase activity was decreased by 22%, Mg^{2+}-ATPase decreased by 24%, $(Na^{+}$-$K^{+})$-ATPase decreased by 35%, while no significant change in Ca^{2+}-ATPase was observed (20,41). Ca^{2+}-free perfusion has also been shown to affect microsomal (sarcoplasmic reticular) and mitochondrial calcium transport (17,42). Mitochondrial calcium binding and calcium uptake were decreased 32% and 23% respectively, after 5 minutes of Ca^{2+}-free perfusion of the isolated rat

heart, while microsomal binding and uptake were unaffected after the same time of calcium deprivation. The authors also reported no significant change in mitochondrial total ATPase and microsomal total and Ca^{2+}-stimulated ATPase activities under the same conditions. It is hard to relate alterations in mitochondrial calcium accumulation to mechanical failure since contractile failure occurred well before mitochondrial changes became significant.

The ability of calcium ion to alter myocardial metabolism is well known (2,3,43). It has been shown that after 4 minutes of Ca^{2+}-free perfusion CP and ATP levels increase by 20% and 10% respectively, in the isolated rat heart. The fact that mechanical activity had ceased rather than Ca^{2+}-depletion *per se*, was the suggested reason for the increase in high energy phosphates, since energy utilization had decreased (18). Twelve minutes of calcium deprivation has been shown to have no effect on rabbit myocardial ATP or CP (30). A recent report on zero calcium-induced changes in myocardial metabolism of isolated rat hearts, has appeared in the literature (43). Similar changes as observed in the previous study, were reported. In addition, Ca^{2+}-free perfusion resulted in decreased glycerol release, increased glycogen stores, decreased CO_2 production and free fatty acid stores, and no change in cAMP levels of the isolated perfused hearts.

In 1966 Zimmerman and Hulsmann first described the curious phenomenon named the "calcium paradox" (28). This

refers to the functional, morphological and biochemical alterations that occur when a Ca^{2+}-free perfused heart is reperfused with normal calcium containing solution.- These first experiments were done on isolated dog and rat hearts and the authors' observations were as follows. Both hypercontracted and severely damaged myocardial cells were seen. Reintroduction of calcium ions caused a loss of electrical and mechanical activity, mitochondrial swelling and the appearance of myoglobin, lactate dehydrogenase, creatine phosphokinase and myosin in the effluent. It was suggested by these investigators, that reperfusion with normal calcium of Ca^{2+}-deprived hearts probably resulted in excessive calcium influx into the isolated hearts. This model has been used by numerous investigators for studying the effects of reperfusion of calcium-deprived hearts, on various myocardial parameters.

The paradoxical influence of calcium ion on myocardial ultrastructure has been studied extensively. In isolated perfused rat hearts at least two minutes of Ca^{2+}-free perfusion is required before reperfusion with normal calcium will produce ultrastructural alterations (20) and after 5 minutes there is damage independent of calcium reperfusion. Reperfusion after 2 - 5 minutes of Ca^{2+}-deprivation causes marked damage, similar to that seen in other species (rabbit, dog) but only after long periods of Ca^{2+}-free perfusion (16,28,30). This damage is believed to be due to "massive calcium influx" (20,28,29,44), though the cause and effect

relationship has yet to be demonstrated. Sarcolemmal disruption and separation of the basement membrane from its plasma membrane have been observed (20,27,35). Derangement of the intercalated disc and broad contracture bands have been seen (20). Mitochondrial and sarcoplasmic reticular swelling in addition to the accumulation of electron dense bodies, surmised to be precipitated calcium salts, in mitochondria (16,20,26,45), are other commonly seen features of the "calcium paradox". Calcium overload is also considered to be a determinant factor in the development of catecholamine-induced myocardial lesions (24,25), while massive calcium influx is considered to cause the ultrastructural damage associated with reperfusion of ischemic myocardium (36,46,47). It should be mentioned here that these severe ultrastructural changes are almost certainly part of the explanation for the irreversible loss of electrical and mechanical activities as well as the biochemical alterations which are commonly seen during the "calcium paradox" phenomenon.

In the rat heart, reperfusion with Ca^{2+}-containing medium following a period of Ca^{2+}-free perfusion induces other physiological derangements as well. The loss of colour and pale mottled appearance of the reperfused heart (21) can be explained by the loss of intracellular myoglobin, a large protein. Other small and large intracellular constituents are also lost into tissue effluents. Potassium ions and lactate dehydrogenase (LDH) are two to note (21). Losses of creatine, AMP and creatine phosphokinase (CPK)

have been observed (18,21,26), but only small amounts of ATP and CP have been found in the effluent. This leads one to question the energy state of the calcium overloaded myocardium. The observation of swollen mitochondria and the fact that calcium can inhibit mitochondrial oxidative phosphorylation (48), suggest that mitochondrial function is impaired. Unpublished observations from this laboratory confirm the above; the degree of impairment ranging from 15% - 50% depression of oxidative phosphorylation (Lee&Dhalla). Further support for this comes from the work of Boink et al.(18). Isolated rat hearts were perfused for 4 minutes with Ca^{2+}-free solution and subsequently reperfused with Ca^{2+}-containing medium. After 30 seconds of reperfusion, electrical activity was lost, irreversible contracture was present, myocardial creatine phosphate (CP) and adenosine-5'-triphosphate (ATP) levels were decreased by 65% and 45% respectively, and creatine, ADP and AMP were increased 15%, 85% and 2800%, respectively. These changes were enhanced with longer periods of reperfusion indicating a severe defect in the machinery which maintains high energy phosphate stores and/or an enhanced energy utilization. Reperfusion of calcium-depleted rabbit hearts also causes a rapid decline in high energy phosphate stores (30).

A more recent report from Zimmerman's laboratory (49) has added a curious and interesting observation to our knowledge of the relationship between high energy phosphates and the "calcium paradox". If isolated rat hearts were perfused for 30 minutes with anoxic medium in the absence of glucose,

high energy phosphate stores were found to be almost completely depleted. Subsequent perfusion for 5 minutes with Ca^{2+}-free solution and reperfusion with normal calcium failed to induce the "calcium paradox". The authors concluded that the "calcium paradox" only occurs in the presence of ATP or oxygen plus substrate, and that mitochondria most likely play a major role because of their ability to accumulate large amounts of Ca^{2+} under these conditions. Intracellular calcium overload has also been shown to initiate a deleterious high energy phosphate deficiency by excessive activation of Ca^{2+}-dependent intracellular ATPases and by impairing the phosphorylating capacity of mitochondria (24,25). In addition, it has been demonstrated that this exhaustion of energy rich phosphates is a crucial point in the etiology of myocardial necrosis produced by excessive calcium (24,50). It seems appropriate at this point to mention the fact that the "calcium paradox", with respect to losses of intracellular components and derangements in high energy phosphate stores and mitochondrial ultrastructure, is not a unique phenomenon to the isolated heart. These same characteristics can be elicited by reperfusing with normal medium a calcium-deprived kidney preparation (51). This suggests that mechanical and electrical activities are not pre-requisites for the induction of the "calcium paradox". However, this point still tends to be a controversial one (45,51,52).

In addition to alterations in sarcolemmal ultrastructure, calcium overload has been shown to affect various sarcolemmal

properties. This data is largely from the work of Dhalla and co-workers (26) on isolated rat hearts. Ten minutes of Ca^{2+}-free perfusion followed by reperfusion with 1.25mM Ca^{2+} for 10 minutes markedly depressed all sarcolemmal enzyme activities when compared to control values; adenylate cyclase (64%↓), Ca^{2+}-ATPase (20%↓), Mg^{2+}-ATPase (43%↓) and Na^{+}-K^{+} ATPase (70%↓). This further supports the statement that calcium overload alters the sarcolemmal membrane. The "calcium paradox" phenomenon is also associated with changes in the subcellular calcium transport systems. Similar changes have been reported in failing hearts (17). Microsomal calcium uptake and binding activity is depressed, in association with depressed ATPase activity, while mitochondrial calcium uptake and binding increase in the isolated, calcium overloaded rat myocardium (17). The fact that mitochondrial calcium accumulation is increased is of particular interest. First of all, it may give a partial explanation for the swollen appearance of mitochondria from calcium overloaded hearts, while also giving credence to the observation that these mitochondria contain large quantities of calcium precipitate. Furthermore, it supplies a mechanism for the inhibition of oxidative phosphorylation by calcium. Finally, energy linked calcium accumulation by mitochondria is known to occur in preference to ATP formation (48). In general however, changes in mitochondrial Ca^{2+} transport appear to be adaptive in nature.

Since many of the cardiac enzyme systems are known

to be pH and temperature sensitive, the effects of these parameters on the occurrence of the "calcium paradox" has also been investigated during the Ca^{2+}-free period, which appears to predispose the heart to calcium overload. Unlike Ca^{2+}-free perfusion at 37°C, perfusion at 4°C causes immediate loss of electrical activity. Even after 30 minutes of Ca^{2+}-free perfusion at 4°C, reperfusion with normal Ca^{2+} at 37°C allows contractile activity to resume and myocardial ultrastructure is protected (45). The authors suggested that during calcium deprivation at 37°C, membrane proteins associated with the "calcium channel" may be lost or their conformation altered in such a way that restitution of calcium would result in massive influx of the ion. The "calcium paradox" is also prevented if the duration of Ca^{2+}-free perfusion at pH 6.8 is shorter than 8 minutes. At pH 8.0 however, only 1½ minutes of Ca^{2+}-free perfusion of isolated rat hearts is required for the occurrence of the "calcium paradox" (44). It was hypothesized that the high H^+ concentration hampers calcium ion movements across cell membranes and/or at the level of the subcellular organelles.

Other ionic variations during the Ca^{2+}-free period have been found to affect the "calcium paradox" phenomenon. A reduction in the Na^+ concentration from 145 mM to 35 mM has been found to delay the initial failure in contractility, augment the recovery of contractile force and protect the heart from severe ultrastructural changes (20). Low Na^+ is believed to delay Ca^{2+} loss during Ca^{2+}-free perfusion (53).

A more recent study has also shown that low Na^+ prevents the changes in microsomal and mitochondrial calcium transport described earlier (17). Yates and Dhalla (20) have demonstrated that reducing Mg^{2+} during the Ca^{2+}-free period delays contractile failure with no effect on recovery, while reducing the K^+ concentration has no effect on the initial contractile depression but diminishes recovery after 3 minutes of Ca^{2+}-free perfusion; this period of Ca^{2+}-depletion normally allowing for recovery in contractility upon reintroduction of the calcium ion. Using perfused rabbit heart intraventricular septum, Crevey et al. (27) found that increasing the Mg^{2+} concentration during Ca^{2+}-free perfusion has a partially protective effect on mechanical recovery and ultrastructural alterations upon reperfusion. They suggest that Mg^{2+} seems able to partially substitute for calcium at membrane superficial levels to support structural and functional properties. If this is indeed the case the question then is, how much Ca^{2+} must be present during the period described as Ca^{2+}-free, to protect the heart from paradoxical calcium influence? The only work to date comes from Langer's laboratory where using rabbit interventricular septum, if 50μM Ca^{2+} is present during what was the Ca^{2+}-free period, the "calcium paradox" is not seen (27).

METHODS

Healthy male Sprague Dawley rats, weighing 250 - 300 grams, were used in the present study. All the animals were kept in environmentally controlled rooms, maintained on standard rat chow *ad libitum* and given free access to water.

A. ISOLATED HEART PREPARATION

Rats were sacrificed by decapitation and their hearts dissected out and placed in ice-cold, oxygenated Krebs-Henseleit (K-H) solution. Atrial, fat and connective tissues were removed and the hearts then arranged for coronary perfusion according to the method of Langendorff, as described elsewhere (54). The hearts were then equilibrated for 15 minutes on K-H medium of the following composition (mM); NaCl,120; $NaHCO_3$,25; KCl,4.8; KH_2PO_4,1.2; $MgSO_4$,1.25; $CaCl_2$,1.25; and glucose, 8.6. The perfusing solution, pH 7.4, was maintained at 37°C (except during experiments with hypothermia) and continually oxygenated with a gas mixture of 95% oxygen and 5% carbon dioxide. Each preparation was electrically paced at 240 beats/minute with a Phipps and Bird 611 square wave stimulator (event duration: 1.5m sec, amplitude: 60 volts). The coronary perfusion rate was 7.8 ml/min. At the onset of each experiment a resting tension of 5 grams was applied to the heart and contractile force (developed tension) monitored on a Gilson polygraph recorder with a force displacement transducer (Grass, FT.03).

For studying the effects of reperfusion, after 5 minutes of Ca^{2+}-free perfusion, with media of altered ionic strength, osmolarity was kept the same as control medium by adding appropriated amounts of sucrose.

At the end of each experimental perfusion period, the coronaries were flushed with 10 ml of ice-cold, Dowex-treated (50W) sucrose (.35M)-histidine (5mM) solution, pH 7.4, so as to wash out the perfusing media. This washout procedure is similar to that employed in previous studies (55,56), in order to minimize contamination from the extracellular compartment. Reperfusion with 10 ml of ice-cold sucrose-histidine solution, of blood-perfused hearts, was found microscopically to completely remove all blood cells from the coronary circulation. No detectable cations were found in the effluent after 6 ml of ice-cold sucrose-histidine medium had been perfused through the heart. There was also no significant change in myocardial cation contents during perfusion with 6-10ml of ice-cold sucrose solution.

Although the control values for myocardial cation contents reported in this study, are comparable to those reported as intracellular cation levels in rat heart (57), it is difficult to rule out the possibility of a small amount of extracellular contamination on the basis of the information at hand. It is for this reason that we have chosen to express our results as myocardial cation contents (μ moles/gm dry heart wt).

After flushing, the hearts were then removed from the perfusion apparatus and visible connective tissue removed.

The ventricles were then blotted gently with gauze, their wet weights determined, and placed in a 100°C oven for 48 hours. After this drying period the hearts were re-weighed and the tissue then processed for determination of Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} contents.

B. CATION DETERMINATIONS

The dried hearts were firstly pulverized and then suspended in 15 mls of Dowex-treated, double distilled, de-ionized water. To this suspension 5 ml of 37 N HCl was added and the resulting solution placed into glass covered test tubes. The tubes were then placed in a boiling water bath for 45 minutes so as to free any bound cations by digesting the tissue. Each sample was then centrifuged for 10 minutes at 10,000 x g and the resulting supernatant decanted off. Aliquots of each sample were then analyzed for cation contents, using a Zeiss Atomic Absorption Spectrophotometer. Recovery varied from 92-102%. No corrections were made in the values reported here.

C. ANALYSIS OF DATA

Each observation was made on at least 4 different preparations. The mean $\pm$ S.E. for each value was calculated and the level of significance of the difference between control and experimental observations was determined by the students paired "t" test. The P values < 0.05 were taken to reflect significance between control and experimental results.

RESULTS

In the first series of experiments (Table 1) the dependency of the "calcium paradox" phenomenon on the calcium concentration was investigated. Reperfusion with normal (1.25mM) calcium, of isolated rat hearts perfused for 5 minutes with 0.10 - 1.25mM Ca^{2+} resulted in complete recovery of contractile force without significant changes ($P > 0.05$) in myocardial cation (Ca^{2+},Mg^{2+},K^{+},Na^{+}) contents. A reduction in calcium concentration to 0.05mM allowed for only 62% recovery in contractile force and significant ($P < 0.05$) elevation in myocardial Ca^{2+} and depression of Mg^{2+} contents, while perfusion with 0.025mM Ca^{2+} resulted in 47% recovery in contractile force and significant ($P < 0.05$) changes in all cations measured, upon reperfusion with normal calcium. Reperfusion after 5 minutes of Ca^{2+}-free exposure, resulted in the inability of the heart to recover its contractile force in addition to producing marked ($P < 0.05$) depressions in Mg^{2+} and K^{+} and dramatic ($P < 0.05$) increases in Ca^{2+} and Na^{+} contents. The relationship between the duration of Ca^{2+}-free perfusion, and contractile force and myocardial cation contents can be appreciated from Table 2. Reperfusion with normal medium after 1 minute of Ca^{2+}-free treatment resulted in full recovery of contractile activity without significantly ($P > 0.05$) altering myocardial cation contents. Longer periods (2,3,4 minutes) of Ca^{2+}-free perfusion were found to steadily depress contractile force recovery upon reperfusion and by 3 minutes of Ca^{2+}-

TABLE 1

INFLUENCE OF 5 MINUTES PERFUSION WITH DIFFERENT CALCIUM CONCENTRATIONS AND REPERFUSION FOR 5 MINUTES WITH 1.25mM CALCIUM ON MYOCARDIAL CATION CONTENTS

		Myocardial cation contents (μ moles/gm dry heart wt)**			
(Ca^{2+})mM	% Recovery of CF	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
1.25(C)	100	3.8 ± 0.36	28.8 ± 0.61	409 ± 18	47.4 ± 3.01
0.50	100	3.8 ± 0.26	29.3 ± 0.99	415 ± 10	48.8 ± 3.36
0.25	100	3.9 ± 0.10	27.1 ± 2.07	405 ± 12	48.3 ± 2.89
0.10	100	4.0 ± 0.44	28.9 ± 2.15	409 ± 11	45.8 ± 4.33
0.05	62 ± 6	6.9 ± 0.52*	24.9 ± 2.10*	400 ± 16	49.2 ± 6.55
0.025	47 ± 7	7.4 ± 0.69*	25.3 ± 1.07*	298 ± 15*	79.6 ± 5.29*
0	0	17.7 ± 0.44*	18.7 ± 0.45*	230 ± 15*	132.9 ± 7.22*

CF: contractile force
C : control (25 minutes perfusion with normal K-H solution)
* $P < 0.05$
** Each value is the mean $\pm$ S.E. of at least 4 experiments

TABLE 2

INFLUENCE OF DIFFERENT TIMES OF Ca^{2+}-FREE PERFUSION AND 5 MINUTES REPERFUSION WITH 1.25mM Ca^{2+} ON MYOCARDIAL CATION CONTENTS

Time of Ca^{2+}-free (min)	% Recovery of CF	Myocardial cation contents (μ moles/gm dry heart wt)**			
		Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
0 (C)	100	3.7±0.13	28.2±0.91	410±14	44.5±2.21
1	106±6	4.0±0.22	26.8±0.73	400±13	45.7±4.37
2	87±5*	4.5±0.31*	26.2±1.28	392±7	59.2±6.11
3	49±8*	5.9±0.25*	24.6±0.83*	370±5*	81.5±5.26*
4	11±5*	9.9±0.50*	22.7±0.45*	320±10*	98.4±9.21*
5	0*	17.6±0.94*	18.7±0.45*	230±15*	132.9±7.22*
10	0*	19.3±0.55*	17.9±0.87*	223±8*	129.9±5.22*

CF: contractile force
C : control (25 minutes perfusion with normal K-H solution)
* $P < 0.05$
** Each value is the mean ± S.E. of at least 4 experiments

free exposure, all cation contents changed significantly ($P < 0.05$) with the reintroduction of Ca^{2+}. After 5 and 10 minutes of calcium-deprivation there was no recovery of contractile force and marked ($P < 0.05$) elevations in myocardial Ca^{2+} and Na^{+}, and depressions in K^{+} and Mg^{2+} contents were apparent.

In order to show the relationship between changes in myocardial cation levels and recovery of contractile force upon reperfusion, the data reported in Tables 1 and 2 were plotted as % change in cation content vs. % change in contractile force recovery. Linear relationships were seen between recovery of contractile force and alterations in myocardial cation contents. The regression coefficients for each plot as well as the % changes in cation content at 50% and 100% depression in recovery of contractile force, are given in Table 3.

The time-course of reperfusion-induced cation alterations after 5 and 10 minutes of Ca^{2+}-free perfusion of the isolated hearts, is represented in Table 4. After 5 minutes of Ca^{2+}-deprivation both myocardial Ca^{2+} and K^{+} contents were significantly ($P < 0.05$) depressed from control, whereas 10 minutes of Ca^{2+}-free perfusion significantly ($P<0.05$) decreased Ca^{2+}, Mg^{2+} and K^{+} contents from their control values, without any change in Na^{+} levels ($P > 0.05$). Significant ($P < 0.05$) increases in myocardial Ca^{2+} and Na^{+} and significant ($P < 0.05$) reduction in Mg^{2+} and K^{+} contents were apparent after 1 minute of reperfusion following both 5 and 10 minutes of Ca^{2+}-free

TABLE 3

RELATIONSHIP BETWEEN % CHANGE IN RECOVERY OF CONTRACTILE FORCE (CF) AND % CHANGE IN MYOCARDIAL CATION CONTENTS

	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
Regression Coefficient	.96	.96	.91	.88
50% recovery of CF	193	85	83	168
0% recovery of CF	267	66	55	235

Cation content is expressed as % of control

TABLE 4

TIME COURSE EFFECTS OF REPERFUSION WITH 1.25mM Ca^{2+} ON MYOCARDIAL CATION CONTENTS AFTER 5 and 10 MINUTES OF Ca^{2+}-FREE PERFUSION

	Myocardial cation contents (μ moles/gm dry heart wt)**			
Time of reperfusion (min)	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
A) 5 min Ca^{2+} free				
control***	3.8±0.26	28.5±0.57	406±8	45.2±2.66
0	2.6±0.18*	27.2±1.28	354±9*	48.3±1.02
1	6.9±0.57*	25.8±0.67*	320±14*	70.4±6.11*
2	10.7±0.58*	22.5±1.17*	281±16*	97.4±4.29*
5	17.6±0.94*	18.7±0.45*	230±14*	132.8±8.22*
10	19.3±0.85*	17.9±1.35*	202±7*	136.45±6.48*
B) 10 min Ca^{2+} free				
control***	3.7±0.24	28.9±0.59	409±9	46.2±1.08
0	2.4±0.11*	25.7±0.95*	315±10*	49.4±2.28
1	7.8±0.39*	25.1±1.11*	289±4*	74.7±5.28*
2	11.7±1.64*	22.4±0.69*	261±5*	97.1±4.85*
5	19.3±0.55*	17.9±0.87*	223±8*	239.9±5.22*
10	20.1±0.98*	14.4±1.01*	180±3*	158.5±5.08*

* $P < 0.05$
** Each value is the mean ± S.E. of at least 4 experiments
*** 25 minutes perfusion with normal K-H solution

perfusion. The directions of change for all 4 cations were enhanced with longer periods of reperfusion, the maximums reached in those hearts exposed to Ca^{2+}-free medium for 10 minutes, followed by 10 minutes reperfusion with normal calcium containing medium.

Low Na^{+} during Ca^{2+} deprivation, has been reported to protect the isolated-perfused rat heart preparation from the occurrence of the "calcium paradox" (20). Cation changes, when hearts perfused with 0 Ca^{2+} in the presence of low (35mM) Na^{+} were reperfused for various times with normal Ca^{2+} containing solution, are presented on Table 5. Five minutes of 0 Ca^{2+}, 35mM Na^{+} perfusion had no effect ($P > 0.05$) on any of the cations measured when compared to control levels. Reperfusion with normal medium for 1 and 2 minutes was without effect ($P > 0.05$) on myocardial cation contents. Longer periods of calcium reintroduction significantly ($P < 0.05$) increased Na^{+} content in a time dependent fashion but Ca^{2+}, Mg^{2+} and K^{+} were unchanged ($P > 0.05$). The contractile force recovered fully in all cases.

Varying degrees of hypothermia during calcium deprivation have also been reported to be protective (45) with respect to the "calcium paradox". The time-course of reperfusion with normal Ca^{2+} after 5 minutes of Ca^{2+}-free perfusion at 21°C, was the subject of the next series of experiments (Table 6). Neither was Ca^{2+}-free exposure at 21°C nor various times of reperfusion at 37°C with Ca^{2+}, capable of altering myocardial cation contents ($P > 0.05$).

TABLE 5

TIME COURSE EFFECTS OF REPERFUSION WITH 1.25mM Ca^{2+} ON MYOCARDIAL CATION CONTENTS AFTER 5 MINUTES OF Ca^{2+}-FREE PERFUSION IN THE PRESENCE OF 35mM Na^{+}

Time of reperfusion (min)	Myocardial cation contents (μ moles/gm dry heart wt)**			
	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
control***	3.7 ± 0.28	26.2 ± 2.99	423 ± 16	47.8 ± 2.98
0	2.9 ± 0.38	28.2 ± 2.79	388 ± 22	49.2 ± 4.89
1	4.0 ± 0.69	27.2 ± 1.09	360 ± 28	54.8 ± 3.77
2	4.2 ± 0.53	30.0 ± 2.00	405 ± 10	60.2 ± 1.77
5	3.8 ± 0.63	25.6 ± 2.10	406 ± 11	66.4 ± 6.48*
10	3.5 ± 0.12	27.1 ± 1.17	409 ± 7	66.2 ± 3.10*

* $P < 0.05$
** Each value is the mean $\pm$ S.E. of at least 4 experiments
*** 25 minutes perfusion with normal K-H solution

TABLE 6

TIME COURSE EFFECTS OF REPERFUSION WITH 1.25mM Ca^{2+} ON MYOCARDIAL CATION CONTENTS AFTER 5 MINUTES OF Ca^{2+}-FREE PERFUSION AT 21°C

Time of reperfusion (min)	Myocardial cation contents (μ moles/gm dry heart wt)**			
	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
control***	3.5±0.28	28.9±1.87	405±26	48.5±3.29
0	2.9±0.38	28.1±0.88	401±9	51.6±1.92
1	3.3±0.44	28.4±1.41	380±15	46.1±3.33
2	3.9±0.23	26.3±0.78	422±16	54.2±3.87
5	3.4±0.67	26.3±2.09	411±9	50.3±7.88
10	3.9±0.40	25.4±2.04	381±7	45.1±2.31

* $P < 0.05$
** Each value is the mean ± S.E. of at least 4 experiments
*** 25 minutes perfusion with normal K-H solution

In order to study the role of Ca^{2+} present in the reperfusion medium, on alterations in myocardial cation contents, calcium-deprived hearts were reperfused with varying concentrations of calcium. The results are shown in Table 7. While reperfusion with 0.025mM Ca^{2+} was without effect ($P > 0.05$), exposure of calcium-deprived hearts to 0.05mM Ca^{2+} resulted in a significant ($P < 0.05$) loss of K^+; the other cations being unaltered ($P > 0.05$). Reperfusion with 0.1mM Ca^{2+} significantly ($P < 0.05$) increased Ca^{2+} and Na^+ and decreased K^+. Concentration dependent increases ($P < 0.05$) in Ca^{2+} and Na^+ and decreases ($P < 0.05$) in Mg^{2+} and K^+ were observed as the calcium concentration of the reperfusion medium rose. The time-course of change in myocardial cation contents when hearts deprived of calcium for 5 minutes are reperfused with low (0.1mM) and high (2.5mM) Ca^{2+} containing solutions, is given in Table 8. When the reperfusion medium contained 0.1mM Ca^{2+}, at least 2 minutes was required before a significant ($P < 0.05$) increase in Ca^{2+} and Na^+ contents could be observed. The increase progressed as the duration of reperfusion lengthened. A significant ($P < 0.05$) decrease in Mg^{2+} occurred after 5 or more minutes of reperfusion, while K^+ was significantly ($P < 0.05$) depressed after the Ca^{2+}-free period and declined steadily over the reperfusion periods studied, in a time-dependent fashion. Reperfusion with 2.5mM Ca^{2+} caused dramatic changes. After 1 minute of calcium reintroduction all cations had significantly ($P < 0.05$) changed in the same direction as in previous studies.

TABLE 7

INFLUENCE OF 5 MINUTES PERFUSION WITH Ca^{2+}-FREE MEDIUM AND REPERFUSION FOR 5 MINUTES WITH DIFFERENT CALCIUM CONCENTRATIONS ON MYOCARDIAL CATION CONTENTS

	Myocardial cation contents (μ moles/gm dry heart wt)**			
(Ca^{2+}) mM	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
control	3.7±.39	28.5±2.16	410±15	45.3±4.99
0	2.6±.18*	27.9±1.18	375±16*	48.9±5.38
0.025	3.0±0.32	26.0±2.13	360±22*	50.3±6.66
0.05	4.5±0.49	25.9±2.58	300±20*	55.8±10.50
0.10	6.9±0.63*	23.7±0.97	260±16*	66.3±8.49*
0.25	7.2±0.46*	21.9±0.68*	281±18*	90.5±6.25*
0.50	8.7±0.41*	22.0±0.94*	241±14*	120.4±11.59*
1.00	13.2±1.07*	21.1±0.79*	229±13*	132.9±6.95*
1.25	17.6±0.44*	18.7±1.45*	230±11*	132.8±7.22*
2.50	22.7±1.34*	20.5±2.80*	200±15*	178.3±5.72*
4.00	27.7±1.59*	15.3±2.19*	159±17*	220.8±16.28*

control: 25 minutes of perfusion with normal K-H solution
0 : 15 minutes of control perfusion plus 5 minutes of Ca^{2+}-free perfusion

* $P < 0.05$

** Each value is the mean ± S.E. of at least 4 experiments

TABLE 8

TIME COURSE EFFECTS OF REPERFUSION WITH 0.1mM and 2.5mM Ca^{2+} ON MYOCARDIAL CATION CONTENTS AFTER 5 MINUTES OF Ca^{2+}-FREE PERFUSION

	Myocardial cation contents (μ moles/gm dry heart wt)**			
Time of reperfusion (min)	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
A) 0.1mM Ca^{2+}				
control***	3.9±0.36	27.7±1.08	398±10	44.4±2.97
0	2.7±0.27*	26.2±0.69	369±8*	46.5±3.21
1	3.1±0.72	27.8±1.05	361±10*	48.9±1.78
2	5.1±0.55*	26.3±0.55	356±7*	50.2±2.23
5	7.0±0.63*	23.7±0.97*	310±11*	66.3±8.49*
10	7.3±1.23*	22.4±1.17*	269±18*	81.6±9.92*
B) 2.5mM Ca^{2+}				
control***	3.8±0.11	28.4±0.72	406±7	45.8±1.72
0	2.5±0.36*	26.1±1.75	351±10*	49.1±1.99
1	10.9±1.56*	24.0±2.06*	320±8*	101.2±9.23*
2	17.21±1.18*	21.4±2.01*	281±17*	145.8±6.11*
5	22.7±0.34*	20.4±2.48*	200±15*	178.3±5.72*
10	23.2±1.09*	16.6±3.21*	108±18*	174.4±6.29*

* $P < 0.05$
** Each value is the mean ± S.E. of at least 4 experiments
*** 25 minutes perfusion with normal K-H solution

These alterations were also enhanced with prolonged times of reperfusion. After 10 minutes of reperfusion with high calcium, relative increases of 6 fold for Ca^{2+} and 4 fold for Na^{+}, and decreases of almost 2 fold for Mg^{2+} and 4 fold for K^{+} were readily apparent.

Since various cations are known to affect calcium movements in the heart (11,53,58), the next series of experiments were carried out to determine whether different concentrations of cations, other than calcium, in the reperfusion medium, can influence the alterations in myocaridal cation contents. For this purpose, the calcium deprived hearts were reperfused with media containing varying amounts of K^{+}, Na^{+} and Mg^{2+}. Time-course of reperfusion with normal Ca^{2+}, in the presence of low (1.5mM) K^{+} and then low (35mM) Na^{+}, of hearts perfused with Ca^{2+}-free medium for 5 minutes, is shown in Table 9. After significant ($P < 0.05$) depression during the calcium free period, 1 minute of reperfusion with media-containing low K^{+} resulted in a significant ($P < 0.05$) increase and decrease in Ca^{2+} and K^{+}, respectively. These changes were again enhanced with longer durations of reperfusion. Mg^{2+} values and Na^{+} content were significantly ($P < 0.05$) different from their respective control values after 1 minute of reperfusion. Increased reperfusion times decreased Mg^{2+} and elevated Na^{+} levels further. A 3-fold decrease in K^{+} is noteworthy, after 10 minutes of reperfusion with media containing low K^{+}. When the reperfusion medium was adjusted so as to reduce the Na^{+} concentration, similar qualitative changes

TABLE 9

TIME COURSE EFFECTS OF REPERFUSION WITH 1.25mM Ca^{2+} IN THE PRESENCE OF 1.5mM K^+ OR 35mM Na^+ ON MYOCARDIAL CATION CONTENTS AFTER 5 MINUTES OF Ca^{2+}-FREE PERFUSION

Time of reperfusion (min)	Myocardial cation contents (μ moles/gm dry heart wt)**			
	Ca^{2+}	Mg^{2+}	K^+	Na^+
A) 1.5mM K^+				
control***	3.8±0.21	29.2±0.99	413±5	45.0±1.91
0	2.6±0.31*	27.7±0.67	360±11*	50.0±3.18
1	8.1±1.79*	25.4±1.11*	248±7*	69.3±9.55*
2	11.9±0.56*	22.7±0.64*	191±8*	105.8±3.87*
5	17.8±0.64*	19.3±0.93*	152±13*	118.7±2.78*
10	16.4±1.38*	18.9±0.69*	145±10*	130.3±8.85*
B) 35mM Na^+				
control***	3.6±0.23	28.7±0.74	390±17	47.4±3.01
0	2.6±0.17*	28.2±1.93	347±7*	48.1±2.21
1	10.9±0.41*	24.7±1.19*	310±9*	49.0±3.88
2	15.5±0.63*	23.1±0.68*	289±14*	51.3±2.13
5	19.4±1.57*	20.4±0.48*	246±8*	60.9±5.27*
10	22.8±0.98*	17.91±0.77*	211±10*	73.3±4.79*

* $P < 0.05$
** Each value is the mean ± S.E. of at least 4 experiments
*** 25 minutes perfusion with normal K-H solution

in Ca^{2+}, Mg^{2+} and K^{+} as those in part A of Table 9 were apparent ($P < 0.05$). At least 5 minutes of reperfusion with low Na^{+} however were required before myocardial Na^{+} content was significantly ($P < 0.05$) increased from its control levels, and 10 minutes of reperfusion only changed ($P < 0.05$) Na^{+} approximately 50%. The next set of experiments investigated the effects of reperfusion with 1.25mM Ca^{2+} in the presence of low (0mM) and high (12mM) Mg^{2+}, after 5 minutes of Ca^{2+}-free perfusion. In Table 10 both reperfusion with low and high Mg^{2+} resulted in similar patterns of change in Ca^{2+}, K^{+} and Na^{+} contents. The increased Ca^{2+}, decreased K^{+} and increased Na^{+} levels were all enhanced ($P < 0.05$) over the reperfusion time. However, the final K^{+} and Na^{+} measurements were higher in hearts perfused with 12mM Mg^{2+} as compared to those exposed to 0 Mg^{2+}. After 10 minutes of reperfusion with high Mg^{2+}, myocardial Mg^{2+} content was significantly ($P < 0.05$) elevated from control, while hearts reperfused with Mg^{2+}-free medium lost ($P < 0.05$) Mg^{2+} steadily over the time-course of reperfusion.

The effects of two known calcium antagonists, verapamil (isoptin) and its methoxy-derivative D_{600}, on myocardial cation contents, are seen on Table 11. Both drugs yielded similar results. One minute of reperfusion significantly ($P < 0.05$) elevated Ca^{2+} and Na^{+} contents and K^{+} were depressed ($P < 0.05$). Two minutes of reperfusion were required before a significant ($P < 0.05$) loss of Mg^{2+} could be demonstrated. The magnitude of change after 10 minutes

TABLE 10

TIME COURSE EFFECTS OF REPERFUSION WITH 1.25mM Ca^{2+} IN THE PRESENCE OF 0mM Mg^{2+} OR 12mM Mg^{2+} ON MYOCARDIAL CATION CONTENTS AFTER 5 MINUTES OF Ca^{2+}-FREE PERFUSION

Time of reperfusion (min)	Myocardial cation contents (μ moles/gm dry heart wt)**			
	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
A) 0.0mM Mg^{2+}				
control***	3.5±0.17	28.3±0.94	404±9	46.1±2.89
0	2.5±0.13*	26.4±0.46	361±5*	49.2±1.17
1	5.2±0.83*	24.4±1.27*	320±17*	53.9±0.86*
2	7.9±1.15*	22.4±0.69*	277±12*	58.2±1.16*
5	17.2±0.48*	18.8±2.63*	211±14*	64.8±0.92*
10	15.6±1.01*	15.8±0.37*	188±14*	71.3±2.13*
B) 12.0mM Mg^{2+}				
control***	3.8±0.37	28.7±0.71	385±15	48.7±2.03
0	2.7±0.16*	26.4±0.68	345±5*	50.0±1.29
1	8.2±0.78*	29.6±0.46	340±16*	61.0±3.39*
2	13.4±0.69*	29.1±0.68	310±29*	82.3±2.07*
5	16.0±0.92*	31.8±2.23	288±9*	99.0±8.74*
10	15.9±1.77*	36.9±2.76*	255±10*	105.9±9.76*

* $P < 0.05$
** Each value is the mean ± S.E. of at least 4 experiments
*** 25 minutes perfusion with normal K-H solution

TABLE 11

TIME COURSE EFFECTS OF REPERFUSION WITH 1.25mM Ca^{2+} IN THE PRESENCE OF 0.1mg/l VERAPAMIL OR 0.1mg/l D_{600} ON MYOCARDIAL CATION CONTENTS AFTER 5 MINUTES OF Ca^{2+}-FREE PERFUSION

Time of reperfusion (min)	Myocardial cation contents (μ moles/gm dry heart wt)** Ca^{2+}	Mg^{2+}	K^+	Na^+
A) 0.1mg/l verapamil				
control***	3.6±0.27	30.9±3.61	390±21	46.2±1.07
0	2.7±0.30*	27.9±1.70	351±16*	49.0±3.27
1	5.3±1.61*	23.8±3.71	310±6*	74.7±1.38*
2	8.2±0.40*	24.1±1.17*	279±6*	89.4±5.07*
5	15.1±1.86*	19.3±2.37*	241±9*	111.2±8.96*
10	17.89±1.31*	18.8±3.70*	211±18*	128.8±9.22*
B) 0.1mg/l D_{600}				
control***	4.0±0.38	28.8±3.09	435±26	49.2±2.09
0	2.7±0.26*	25.0±1.01	369±11*	49.1±2.33
1	6.9±0.91*	25.2±2.55	320±10*	75.9±5.47*
2	14.3±1.19*	21.1±1.97*	274±16*	101.8±9.88*
5	19.2±0.57*	20.0±2,13*	251±12*	140.1±15.29*
10	20.1±2.19*	19.2±1.17*	206±14*	131.7±9.53*

* $P < 0.05$
** Each value is the mean ± S.E. of at least 4 experiments
*** 25 minutes perfusion with normal K-H solution

of reperfusion does not appear different from those under the same condition in Table 3A, which is reperfusion with normal Ca^{2+} after 5 minutes of Ca^{2+}-deprivation. In the last series of experiments (Table 12) reperfusion after 5 minutes of Ca^{2+}-free perfusion, was carried out with media containing normal Ca^{2+} and propranolol, a well known β-receptor blocker. The results obtained are qualitatively and quantitatively similar to those in the presence of verapamil and D_{600}.

TABLE 12

TIME COURSE EFFECTS OF REPERFUSION WITH 1.25mM Ca^{2+} IN THE PRESENCE OF 1.0mg/l PROPRANOLOL ON MYOCARDIAL CATION CONTENTS AFTER 5 MINUTES OF Ca^{2+}-FREE PERFUSION

Time of reperfusion (min)	Myocardial cation contents (μ moles/gm dry heart wt)**			
	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
control***	3.4±0.22	26.9±2.01	415±9	48.2±1.88
0	2.4±0.38*	27.2±1.03	367±13*	49.1±0.91
1	5.0±0.19*	25.4±0.96	321±13*	70.9±5.06*
2	9.1±0.48*	21.1±1.77*	279±7*	99.6±6.85*
5	16.4±0.92*	21.2±2.00*	230±9*	120.2±10.29*
10	20.2±1.67*	19.0±2.30*	220±16*	134.7±8.23*

* $P < 0.05$
** Each value is the mean ± S.E. of at least 4 experiments
*** 25 minutes perfusion with normal K-H solution

DISCUSSION

In the present study, myocardial cation contents of isolated rat hearts perfused under various conditions, were determined. The control values (expressed as μ moles/ gm dry heart wt) of hearts perfused for 15-25 minutes with normal Krebs-Henseleit solution, are essentially similar to those reported by Polimeni (57) for rat heart.

When equilibrated preparations were perfused with Ca^{2+}-free media, contractile activity (developed tension) declined and was completely absent within 20 seconds; in good agreement with earlier reports (16,20,21). It has been demonstrated that if this period of Ca^{2+}-deprivation lasts for 5 or 10 minutes, there are significant changes in myocardial cation contents. Potassium ion content of the heart decreases, while Na^{+} shows a slight but non-significant increase, similar to that seen by other investigators (13,27). The depression seen in calcium levels is support for the contention that this experimental procedure results in calcium depletion. Previously, 2-10 minutes of calcium-free exposure has been found to result in a reduction in calcium levels of rat heart homogenates (16), and longer periods of Ca^{2+}-deprivation of rabbit hearts results in similar depression of calcium contents (30). It has been suggested that the sources of the lost calcium are a contractile calcium compartment, lost in 2.5-3 minutes, and a structural calcium compartment, which is depleted only after 10 minutes Ca^{2+}-free perfusion

of isolated rat hearts (16).

Reperfusion for 5 minutes with 1.25mM Ca^{2+} after 5 minutes of Ca^{2+}-free perfusion, resulted in total cardiac contracture and the hearts became pale and mottled. These findings confirm the initial observations of Zimmerman and Hulsmann (21,28) who termed this response the "calcium paradox" and attributed the phenomenon to excessive calcium influx. The results reported here demonstrate that this condition is associated with a marked increase in myocardial Ca^{2+} and Na^{+} contents, while Mg^{2+} and K^{+} levels are significantly depressed from control values. Additions of calcium (0.025-0.050mM) during what was the Ca^{2+}-free period, was found to partially protect the myocardium from these changes, whereas perfusion with 0.10 - 1.25mM calcium prevented the changes associated with reperfusion. The explanation for the protective effect of calcium could be that the presence of Ca^{2+} prevented the permeability changes in the sarcolemmal membrane,which are believed to predispose the hearts to excessive calcium influx upon the reintroduction of normal calcium (21,31,43).

Not only was the concentration of extracellular calcium critical for demonstrating changes in recovery of contractile force and myocardial cation contents due to reperfusion, but these parameters were also dependent on the time of Ca^{2+}-free perfusion. Previous reports have indicated that at least 2 minutes of Ca^{2+}-free exposure is necessary before recovery is affected by reperfusion and anywhere from 3-5 minutes of

Ca^{2+}-deprivation required to maintain zero contractile force generation upon reperfusion (20,28,44,45,52). In the present study at least 5 minutes of Ca^{2+}-free perfusion was required to completely inable the myocardium to generate contractile force upon reperfusion, while an impairment in recovery of contractile force was apparent after 2-4 minutes of Ca^{2+}-free perfusion. Differences, though slight, can be explained by small dissimilarities in the experimental design from lab. to lab. Alterations in the cation contents upon reperfusion, were also dependent on the duration of Ca^{2+}-depletion. The most profound jump in calcium content occurred in those hearts reperfused after 4-5 minutes of Ca^{2+}-free perfusion, the point where reperfusion with normal calcium completely prevented the recovery of contractile force. The changes which occurred in Mg^{2+}, K^{+} and Na^{+} contents were also dependent on the duration of Ca^{2+}-free exposure, but it is hard to interpret or relate these changes with respect to contractile force alterations. It should be noted that various enzyme systems are dependent on these cations (15) and the imbalance of Na^{+} and K^{+} may partially account for the loss of electrical activity reported earlier (30). However, the changes in myocardial cation contents may be just a reflection of sarcolemmal disruption and movement of the cations along their concentration gradients.

The magnitude, but not the directions of change in myocardial cation contents was also found to be related to the duration of reperfusion with normal calcium. It was

interesting to note that 5 minutes reperfusion after 4 minutes of Ca^{2+}-deprivation, elevated myocardial calcium approximately 2.5 fold with 10% of the control contractile force remaining, while 1 minute reperfusion after 5 and 10 minutes of Ca^{2+}-free exposure only increased calcium content 1.75 and 2.0 fold respectively, but there was no mechanical activity observed. It appears that the "calcium paradox" is a complicated phenomenon and not simply expressed as calcium overload. In fact, the recovery of contractile force upon reperfusion appeared to be linearly related to increases in Ca^{2+} and Na^{+}, and decreases in Mg^{2+} and K^{+} contents of the myocardium. However, these relationships are based on a limited range of changes in contractile force and cation contents and thus a great deal of caution should be exercised in deriving conclusions from these results. A more extensive investigation seems warrented before any definitive statement on the significance of these observations, can be made.

A reduction in the Na^{+} concentration from normal (145mM) to 35mM, during the Ca^{2+}-free perfusion period, has been shown capable of protecting the myocardium from the occurrence of the "calcium paradox" when calcium is reintroduced (20). Reduced loss of calcium during the period of Ca^{2+}-deprivation is the suggested mechanism of protection. Even though the myocardial calcium level appeared to be higher after 5 minutes of Ca^{2+}-free perfusion in the presence of low Na^{+}, than after 5 minutes of Ca^{2+}-free exposure with normal Na^{+} concentration, there was no statistically significant difference. On the other hand, there was also no significant

difference between the values of myocardial calcium after 0 Ca^{2+}, low Na^{+} perfusion and their paired controls. This is consistent with the view that a small amount of sarcolemmal calcium may be retained during Ca^{2+}-free perfusion with low Na^{+}, and this may serve as a protective mechanism against the dramatic changes associated with reperfusion. At any rate, over the entire reperfusion period there were no changes in myocardial Ca^{2+}, K^{+}, and Mg^{2+} contents and 100% recovery of contractile force. The slight increase in Na^{+} content after 2 minutes of reperfusion, seems to indicate that the myocardium can handle a moderate elevation in Na^{+} with respect to the maintenance of normal contractile function.

Hypothermia during Ca^{2+}-free perfusion, has also been shown to prevent the "calcium paradox" phenomenon in isolated rat hearts (45). In the present study, Ca^{2+}-free perfusion at room temperature (21°C) was without effect on myocardial cation contents and reperfusion resulted in preservation of control heart cation levels and 100% recovery of contractile force. The lack of Ca^{2+} and K^{+} loss during the 5 minute Ca^{2+}-free period seems to be due to preservation of the sarcolemmal permeability characteristics, since excessive calcium influx and overload appeared to be completely prevented. If we consider the models even remotely similar, then this data further support the use of hypothermia during coronary-bypass surgery. It should be mentioned that reduced pH during calcium-free perfusion is also protective with

respect to induction of the "calcium paradox" (44), and the relationship between this phenomenon and myocardial cation contents should be a subject for further investigation.

The dependency of the "calcium paradox" and associated cation content alterations, on the amount of calcium influx, was tested in the second part of this study. Regardless of the type of reperfusion intervention employed in this report, as long as the 5 minute Ca^{2+}-free perfusion period was left unaltered, there was absolutely no recovery of contractile force when calcium was reintroduced. It appears that whatever occurs during the period of Ca^{2+}-depletion, is drastic enough to abolish the ability of the myocardium to regain any contractility. This should not yet be taken to mean that other interventions are incapable of reversing the effects of 5 minutes of calcium-depletion.

Previously published reports have demonstrated that perfusion with low Ca^{2+} and high Ca^{2+} results in reduced (35) and increased (16,58) contractile force generation of normal myocardium, respectively. These observations can be attributed to the differences in calcium influx upon excitation of the myocardium, due to the respective concentration gradients present. Changes in myocardial cation contents were found to be dependent on the concentration of calcium in the reperfusion medium. It was interesting to note that the K^+ content was the most sensitive and Mg^{2+} most resistant to Ca^{2+}-induced changes, indicating differences in the sensitivities of these two pools with those of Na^+ and Ca^{2+}. A critical calcium

concentration of 0.25mM in the reperfusion medium was required before significant changes in all myocardial cations were observed. It is possible that this concentration of calcium in the reperfusion medium is large enough to cause further sarcolemmal damage in the Ca^{2+}-depleted hearts, such that all the cations can move according to their concentration gradients. On the other hand, this extracellular concentration of calcium may be required to activate various sarcolemmal exchange mechanisms, accounting for the alterations in myocardial cation contents. The most dramatic changes occurred when reperfusion with high (2.5,4.0mM) Ca^{2+} was carried out after 5 minutes of Ca^{2+}-free perfusion. Under these conditions damage is probably so severe that cation movement may be almost unimpaired, but no data on this is as yet available. Studies on myocardial ultrastructure, primarily sarcolemma, under these conditions may support this suggestion in part. Cation content changes when reperfusion was carried out in the presence of low (0.1mM) and high (2.5mM) calcium, were also demonstrated to be time dependent. Reperfusion with high calcium however, allowed Ca^{2+}, Mg^{2+} and Na^{+} changes to occur earlier, while significant depression in K^{+} was immediate in both cases, but of a greater magnitude in those hearts reperfused with 2.5mM Ca^{2+}.

The effect of normal Ca^{2+} reperfusion with low Na^{+} (35mM), on myocardial cation contents, is similar to that when reperfusion with high Ca^{2+} is performed. Both experimental conditions elevate calcium levels over those myocardial calcium

values from hearts reperfused with normal Krebs-Henseleit solution. The increased myocardial calcium seen with low Na^+ reperfusion can be explained when one realizes that by reducing Na^+, the competition for entry via the slow channels is swung favouring Ca^{2+} influx (27,59). This point is further emphasized by the fact that perfusion with normal Ca^{2+}, low Na^+ medium produces a positive inotropic action on normally contracting hearts (58). The damped increase in myocardial Na^+ content is probably due to a reduction in the concentration gradient favouring Na^+ influx. Of interest is the observation that K^+ loss is also retarded in comparison to the values achieved with high Ca^{2+} reperfusion. Whether a link between K^+ efflux and Na^+ influx is present, is hard to say.

Potassium and Mg^{2+} are also believed to compete with Ca^{2+} at the level of the sarcolemmal membrane (24,25). Reperfusion with low (1.5mM) K^+ elevated myocardial calcium content to the same degree as that in hearts reperfused with normal medium, both after 5 minutes of Ca^{2+}-free perfusion. K^+ loss however, was more pronounced than in early studies, probably due to the enhanced concentration gradient for K^+ efflux. Similar changes in calcium content, to those of low K^+ reperfused hearts were observed whether the reperfusion medium was altered so as to increase (12mM) or decrease (0mM) Mg^{2+} concentration. Myocardial Mg^{2+} content was markedly depressed in hearts reperfused with 0 Mg^{2+} while reperfusion for 10 minutes with high Mg^{2+}, increased the myocardial Mg^{2+}

content; the effects again appeared to be concentration gradient dependent. The fact that all these conditions resulted in similar alterations in Ca^{2+} content, suggests that either the competition sites are lost or the changes in calcium are predisposed during the Ca^{2+}-free period. Since reperfusion with three known Ca^{2+} antagonists (24,25), propranolol, verapamil and D_{600}, was also unable to ater the increase in myocardial calcium content, it appears as if the latter of the two hypotheses is viable. It should be mentioned however, that even though the concentrations of the three calcium antagonists used have been found to depress contractile force generation in normally beating isolated rat hearts (unpublished observations, Dhalla and Yates), a full dose-response study will have to be undertaken before any conclusive statement can be made. For the present, the results seem to implicate membrane integrity, with respect to calcium movements, in the genesis of the "calcium paradox".

CONCLUSIONS

In this study,the effects of various interventions on myocardial cation contents during induction of the "calcium paradox" in isolated rat hearts, were investigated. From the data obtained in this study, the following conclusions are drawn:

a) Calcium-free perfusion results in decreased myocardial Ca^{2+} and K^{+}, while reperfusion results in increased Ca^{2+} and Na^{+}, and decreased Mg^{2+} and K^{+} contents.

b) Changes in recovery of contractile force are well correlated to changes in all the cations studied, but further investigation must be undertaken in order to establish the significance of these observations.

c) The "calcium paradox" phenomenon is heavily dependent on time and the concentration of calcium, both during the Ca^{2+}-free and reperfusion periods.

d) Interventions such as low Na^{+} and hypothermia during Ca^{2+}-free perfusion, which are known to prevent the "calcium paradox", have also been found to prevent the alterations in myocardial cation contents before and during reperfusion with normal calcium.

e) Minimal modification of changes in the myocardial cation contents during reperfusion, was achieved by alterations in the composition of the reperfusion medium with respect to Na^{+}, K^{+} and Mg^{2+}, or by agents which are known to depress calcium influx.

f) The results of this study demonstrate that reperfusion of Ca^{2}-deprived hearts and the failure of contractile activity which results, may not be simply a reflection of intracellular calcium overload and its associated effects but alterations in the content of the other myocardial cations may also play a significant role in this regard.

REFERENCES

1. Wollenberger, A. 1949. The energy metabolism of the failing heart and the metabolic action of the cardiac glycosides. J. Pharm. Exp. Ther. 97: 311-352.

2. Olsen, R.E. and D.A. Barnhorst. 1973. The control of energy production and utilization in cardiac muscle. In Myocardial Metabolism (N.S. Dhalla, ed.) pp 11-30, University Park Press, Baltimore.

3. Opie, L.H. 1969. Metabolism of the heart in health and disease. Amer. Heart J. 76: 685-698, 77: 100-122, 77: 383-410.

4. Pool, P.E. 1974. The biochemical basis of heart failure. In The Myocardium: Failure and Infarction (E. Braunwald, ed.), pp 49-56, H.P. Publishing Co., Ltd., New York.

5. Dhalla, N.S. 1976. Editorial: Involvement of membrane systems in heart failure due to calcium overload and deficiency. J. Mol. Cell. Cardiol. 8: 661-667.

6. Ringer, S. 1883. A further contribution regarding the influence of different constituents of the blood on the contraction of the heart. J. Physiol. 4: 29-42.

7. Heilbrunn, L.V. and F.J. Wiercinski. 1947. The action of various cations on muscle protoplasm. J. Cell. Comp. Physiol. 29: 15-32.

8. Niedergerke, R. 1955. Local muscular shortening by intracellularily applied calcium. J. Physiol. 128: 12-13.

9. Niedergerke, R. 1956. The "staircase" phenomenon and the action of calcium on the heart. J. Physiol. 134: 569-583.

10. Ebashi, S. and M. Endo. 1968. Calcium and muscle contraction. Prog. Biophys. Mol. Biol. 18: 123-183.

11. Langer, G.A. 1968. Ion fluxes in cardiac excitation and contraction and their relation to myocardial contractility. Physiol. Rev. 48: 708-757.

12. Olson, R.E. 1971. Introduction: In Calcium and the Heart (P. Harris, L. Opie eds.), pp 1-23, Academic Press, London.

13. Langer, G.A. 1973. Excitation-contraction coupling. Ann. Rev. Physiol. 35: 55-86.

14. Dhalla, N.S., P.K. Das, and G.P. Sharma. 1977. Subcellular basis of cardiac contractile failure. J. Mol. Cell. Cardiol. in press.

15. Dhalla, N.S., A. Ziegelhoffer, and J.A.C. Harrow. 1977. Regulatory role of membrane systems in heart function. Can. J. Physiol. Pharm., 55.

16. Tomlinson, C.W., J.C. Yates, and N.S. Dhalla. 1974. Relationship among changes in intracellular calcium stores, ultrastructure and contractility of myocardium. In: Recent Advances in Studies on Cardiac Structure and Metabolism, Vol. 4 (N.S. Dhalla, ed.), pp 331-345, University Park Press, Baltimore.

17. Lee, S.L. and N.S. Dhalla. 1976. Subcellular calcium transport in failing hearts due to calcium deficiency and overload. Amer. J. Physiol. 231: 1159-1165.

18. Boink, A.B.T.J., T.J. Ruigrok, and A.N.E. Zimmerman. 1976. Changes in high energy phosphate compounds of isolated rat hearts during Ca^{2+}-free perfusion and reperfusion with Ca^{2+}. J. Mol. Cell. Cardiol. 8: 973-979.

19. Weiss, O.L., B. Surawicz, and I. Ruberstein. 1966. Myocardial lesions of calcium deficiency causing irreversible myocardial failure. Amer. J. Pathol. 48: 653-666.

20. Yates, J.C. and N.S. Dhalla. 1974. Structural and functional changes associated with failure and recovery of hearts after perfusion with Ca^{2+}-free medium. J. Mol. Cell. Cardiol. 7: 91-103.

21. Zimmerman, A.N.E., W. Daems, W.C. Hulsmann, J. Snijder, E. Wisse, and D. Durrer. 1967. Morphological changes of heart muscle caused by successive perfusion with calcium-free and calcium-containing solutions (calcium paradox) Cardiovas. Res. 1: 201-209.

22. Lossnitzer, K. and E. Bajusz. 1974. Water and electrolyte alteration during the life-course of the B10 14.6 Syrian golden hamster. A disease model of a hereditory cardiomyopathy. J. Mol. Cell. Cardiol. 6: 163-177.

23. Jennings, R. and C.E. Ganote. 1974. Structural changes in myocardium during acute ischemia. Circ. Res., Suppl. 3 (Vol. 34), Sept.

24. Fleckenstein, A., J. Janke, H.J. Doring, and O. Leder. 1974. Myocardial fibre necrosis due to intracellular Ca-overload-a new principle in cardiac pathophysiology. In: Myocardial Biology (N.S. Dhalla ed.) pp. 563-580, Urban and Schwarzenberg, Munchen.

25. Fleckenstein, A., J. Janke, H.J. Doring, and O. Pachinger. 1973. Ca-overload as the determinant factor in the production of catecholamine-induced cardiac lesions. In: Cardiomyopathies (E. Bajusz, G. Rona eds) pp. 455-466, University Park Press, Baltimore.

26. Dhalla, N.S., C.W. Tomlinson, J.N. Singh, S.L. Lee, D.B. McNamara, J.A.C. Harrow and Y.C. Yates. 1976. Role of sarcolemmal changes in cardiac pathophysiology. In: Recent Advances in Cardiac Structure and Metabolism (Vol. 9) (E.P. Roy, N.S. Dhalla eds) pp. 377-393, University Park Press, Baltimore.

27. Crevey, B.J., G.A. Langer, and J.S. Frank. 1977. Role of Ca^{2+} in the maintenance of rabbit myocardial cell membrane structural and functional integrity. Amer. J. Physiol., in press.

28. Zimmerman, A.N.E. and W.C. Hulsmann. 1966. Paradoxical influence of calcium ions on the permeability of the cell membranes of the isolated rat heart. Nature 211: 646-647.

29. Lee, Y.C.P. and M.B. Visscher. 1970. Influx of calcium into rabbit myocardium in relation to its ionic environment. Proc. Nat-Acad. Sci. 66: 603-606.

30. Lee, Y.C.P. and M.B. Visscher. 1970. Perfusate cations and contracture and Ca,Cr,PCr and ATP in rabbit myocardium. Amer. J. Physiol. 219: 1637-1641.

31. Bailey, L.E. and H.A. Sures. 1971. The effect of ouabain on the washout of and uptake of calcium in the isolated cat heart. J. Pharm. Exp. Ther. 178: 259-270.

32. Shine, K.I., S.D. Serena, and G.A. Langer. 1971. Kinetic localization of contractile calcium in rabbit myocardium. Amer. J. Physiol. 221: 1408-1417.

33. Mines, G.R. 1913. On the functional analysis by the action of electrolytes. J. Physiol. 46: 188-235.

34. Locke, F.S. and O. Rosenheim. 1907. Contribution to the physiology of the isolated heart. The consumption of dextrose by mammalian cardiac muscle. J. Physiol. 36: 205-220.

35. Muir, A.R. 1967. The effects of divalent cations on the ultrastructure of the perfused rat heart. J. Anat. 101: 239-261.

36. Schaper, J., F. Hehrlein, M. Schlepper, and K-V. Thiedemann. 1977. Ultrastructural alterations during ischemia and reperfusion in human hearts during cardiac surgery. J. Mol. Cardiol. 9: 175-189.

37. Rand, R.P. and S. Sengupta, 1972. Cardiolipin forms hexogonal structures with divalent cations. Biochim. Biophys. Acta. 255: 484-492.

38. Singer, S.J. and G.L. Nicholson. 1972. The fluid mosaic model of the structure of cell membranes. Science 175: 720-731.

39. Frankenhaeuser, B. and A.L. Hodgkin. 1957. The actions of calcium on the electrical properties of squid axons. J. Physiol. 137: 218-244.

40. Morrill, G.A. H.R. Kaback, and E. Robbins. 1964. Effect of calcium on intracellular sodium and potassium concentrations in plant and animal cells. Nature 204: 641-642.

41. Lee, S.L. and N.S. Dhalla. 1973. (Abstract) Fed. Proc. 32, 434.

42. McNamara, D.B., J. Singh, and N.S. Dhalla (Abstract) Fed. Proc. 32, 709.

43. Dhalla, N.S., J.C. Yates, and V. Proveda. 1977. Calcium linked changes in myocardial metabolism in the isolated perfused rat heart. Can. J. Physiol. Pharmacol. 55: 925-933.

44. Bielecki, K. 1969. The influence of changes in pH of the perfusion fluid on the occurrence of the calcium paradox in the isolated rat heart. Cardiovas. Res. 3: 268-271.

45. Holland, C.E. and R.E. Olson, 1975. Prevention by hypothermia of paradoxical calcium necrosis in cardiac muscle. J. Mol. Cell. Cardiol. 7: 917-928.

46. Shen, A.C. and R.B. Jennings. 1972. Kinetics of calcium accumulation in acute myocardial ischemic injury. Amer. J. Pathol. 67: 441-452.

47. Shen, A.C. and R.B. Jennings. 1972. Myocardial calcium and magnesium in acute ischemic injury. J. Pathol. 67: 417-433.

48. Lehninger, A.L. 1970. Mitochondria and calcium transport. Biochem. J. 119: 129-318.

49. Ruigrok, T.J.C., A.B.T.J. Boink, and A.N.E. Zimmerman. 1977. Influence of ATP or oxygen plus substrate on the occurrence of the calcium paradox. In: Recent Advances in Cardiac Structure and Metabolism (Kobyashi, T., T. Sano, N.S. Dhalla eds.) pp 267-269, University Park Press, Baltimore.

50. Nirdlinger, E.L. and P.O. Bramante. 1974. Subcellular myocardial ion shifts and mitochondrial alterations in the course of isoproterenol-induced cardiopathy in rat. J. Mol. Cell. Cardiol. 6: 49-60.

51. Nozick, J.H., A.N.E. Zimmerman, P. Pool, and B.J. Mankowitz. 1971. The kidney and the calcium paradox. J. Surg. Res. 11: 60-67.

52. Ruigrok, T.J.C., F.J.A. Burgersdijk, and A.N.E. Zimmerman. 1975. The calcium paradox: a reaffirmation. Eur. J. Cardiol. 3: 59-63.

53. Reuter, H. and N. Seitz. 1968. The dependence of efflux of calcium from cardiac muscle on temperature and external ionic composition. J. Physiol. 195: 451-470.

54. Tomlinson, C.W. and N.S. Dhalla. 1976. Alterations in myocardial function during bacterial infective cardiomyopathy. Amer. J. Physiol. 37: 373-381.

55. Tomlinson, C.W. and N.S. Dhalla. 1973. Excitation-contraction coupling in heart: IX. Changes in the intracellular stores of calcium in failing hearts due to substrate lack and oxygen. Cardiovas. Res. 8: 470-476.

56. Tomlinson, C.W. and N.S. Dhalla. 1972. Myocardial contractility. II. Effect of changes in cardiac function on the subcellular distribution of calcium in the isolated rat heart. Can. J. Physiol. Pharm. 50: 853-859.

57. Polimeni, P.I. 1974. Cardiac electrolytes and water in thyroparathyroidectomized rat. J. Mol. Cell. Cardiol. 6: 531-541.

58. Legato, M.J. D. Spiro, and G.A. Langer. 1968. Ultrastructural alterations produced in mammalian myocardium by variation in perfusate ionic composition. J. Cell. Biol. 37: 1-12.

59. Langer, G.A., E. Sato, and M. Seraydarian. 1969. Calcium exchange in a single layer of rat cardiac cells studied by direct counting of cellular activity of labelled calcium. Circ. Res. 24: 589-597.