# SUBCELLULAR BASIS OF THE CARDIOTOXIC EFFECTS OF COBALT, NICKEL AND MANGANESE

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by
James A.C. Harrow
Department of Physiology
Faculty of Medicine
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"SUBCELLULAR BASIS OF THE CARDIOTOXIC EFFECTS OF COBALT, NICKEL AND MANGANESE"

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JAMES A.C. HARROW

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## ABSTRACT

The effects of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on the contractile force of the isolated rabbit heart and the activities of the rabbit heart myofibrils, mitochondria, fragments of sarcoplasmic reticulum (microsomes) and sarcolemma were examined in order to establish the sites of action of these divalent cations. $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ in concentrations from 0.1 to 1 mM were found to depress the magnitude and the rate of change of contractile force developement to varying degrees; however, $Ni^{++}$ was found to be more effective than $Co^{++}$ or $Mn^{++}$. Both $Co^{++}$ and $Ni^{++}$, but not $Mn^{++}$, increased the resting tension of these hearts. Electron microscopic examination of the $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ treated hearts revealed no ultrastructural changes due to perfusion with these divalent cations.

Although $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were found to increase ATP hydrolysis by myofibrils, these cations were less active than $Ca^{++}$ or $Mg^{++}$. Myofibrillar ATP hydrolyzing activities due to $Mg^{++}$ ATPase and $Ca^{++}$ - stimulated ATPase were 0.032 and 0.186 μ moles Pi/mg protein/min respectively. Both myofibrillar $Mg^{++}$ ATPase and $Ca^{++}$ - stimulated ATPase activities were inhibited to varying degrees by 0.05 to 1 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, except $Mg^{++}$ ATPase activity was not affected by 0.05 mM $Ni^{++}$ significantly. The order of potency for inhibiting $Mg^{++}$ ATPase was $Mn^{++} > Co^{++} > Ni^{++}$ whereas that for $Ca^{++}$ - stimulated ATPase was $Ni^{++} > Co^{++} > Mn^{++}$. In contrast to the depressant effect of $Co^{++}$ and $Ni^{++}$, the inhibitory actions of $Mn^{++}$ were not apparent at 0.25 to 1 mM concentrations of Mg ATP.

ATP hydrolysis by mitochondria was stimulated by $Ca^{++}$, $Mg^{++}$, $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ in 0.05 to 4 mM concentrations. The mitochondrial ATPase activity (1 μ mole Pi/mg protein/min) in the presence of $Mg^{++}$ was significantly depressed by 0.25 - 4 mM $Ni^{++}$ and 1 - 4 mM $Co^{++}$ or $Mn^{++}$. Mitochondrial calcium binding (52 n moles/mg protein/5 min) and uptake (131 - 186 n moles/mg protein/5 min, under different experimental conditions) activities were depressed to varying degrees

by 0.01 - 1 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The order of potency for inhibiting mitochondrial calcium accumulation was $Ni^{++} > Co^{++}$ and $Mn^{++}$. Mitochondrial ADP : O ratio and RCI were decreased by 0.05 - 0.10 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$.

$Co^{++}$, $Ni^{++}$ and $Mn^{++}$, like $Ca^{++}$ and $Mg^{++}$, were found to stimulate microsomal ATP hydrolysis. Both $Mg^{++}$ ATPase (1.41 μ moles Pi/mg protein/min) and $Ca^{++}$ - stimulated ATPase (0.44 μ moles Pi/mg protein/min) activities were decreased in the presence of 0.25 - 4 mM $Co^{++}$, $Ni^{++}$, $Mn^{++}$ and the order of potency was $Ni^{++} > Co^{++} > Mn^{++}$. Microsomal calcium binding (43 n moles/mg protein/5 min) was not affected by 0.1 - 2 mM $Co^{++}$, $Ni^{++}$ or $Mn^{++}$. On the other hand, microsomal calcium uptake (1064 n moles/mg protein/5 min) activity was decreased to a varying degree by 0.25 - 2 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ and the order of potency was $Ni^{++} > Mn^{++} > Co^{++}$. The inhibitory effects of these divalent cations on calcium uptake activity were observed at 10 - 100 μ M concentrations of calcium.

The ability of sarcolemma to hydrolyze ATP was stimulated by 0.1 to 4 mM concentrations of $Ca^{++}$, $Mg^{++}$, $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The sarcolemmal $Ca^{++}$ ATPase (22.8 μ moles Pi/mg protein/hr) and $Mg^{++}$ ATPase (21.6 μ moles Pi/mg protein/hr) activities were depressed by 0.25 to 4 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ and the order of their potency was $Ni^{++} > Co^{++}$ and $Mn^{++}$. The sarcolemmal $Na^{+}$ - $K^{+}$ ATPase activity (9.4 μ moles Pi/mg protein/hr) was also decreased by 0.10 to 4 mM concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The sarcolemmal calcium binding in the presence of 0.1 mM $Ca^{++}$ (98 n moles/mg protein/5 min) was depressed by 0.25 mM or higher concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ whereas that in the presence of 1.25 mM $Ca^{++}$ (772 n moles/mg protein/5 min) was decreased by 2 - 4 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The sarcolemmal adenylate cyclase activities in the absence (124 p moles cyclic AMP/mg protein/min) and presence of 2 mM NaF (517 p moles cyclic AMP/mg protein/min) were decreased by 0.1 to 4 mM $Co^{++}$ or $Ni^{++}$ and stimulated by 0.1 to 4 mM, $Mn^{++}$.

These results clearly indicate that $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ are capable of altering the functional activities of cardiac myofibrils, mitochondria, sarcoplasmic reticulum and sarcolemma. Changes in the functions of subcellular components in

addition to sarcolemmal alterations may participate in eliciting cardiodepressant actions depending upon the concentrations of these divalent cations. Displacement of calcium from some sarcolemmal sites by these divalent cations may make less calcium available for contraction. In addition these cations may enter the cell and act as a "false - coupler" of the events leading from excitation to contraction. On the basis of sensitivities of different subcellular components to divalent cations, ii is concluded that changes in mitochondrial calcium transport activities may play a predominant role in modifying the contractile activity due to $Co^{++}$ and $Ni^{++}$ whereas changes in mitochondrial oxidative phosphorylation and myofibrillar ATPase activities may be of primary importance for the cardiodepressant effect of $Mn^{++}$.

# I. INTRODUCTION AND STATEMENT OF THE PROBLEM

Various cellular components such as sarcolemma, mitochondria, sarcoplasmic reticulum and myofibrils are generally considered to be intimately involved in heart function and metabolism. The participation of these organelles in contractile events is based on their ability to regulate the intracellular concentration of calcium which has been recognized to play a central role in the cardiac excitation - contraction coupling and relaxation processes. For example, various enzyme systems such as adenylate cyclase, $Na^{+}$ - $K^{+}$ ATPase and $Ca^{++}/Mg^{++}$ ATPase in sarcolemma are believed to be involved in the regulation of calcium movements across the cell membrane. Furthermore, sarcoplasmic reticulum and possibly mitochondria are known to accumulate calcium by energy dependent mechanism and in addition mitochondria generates the major portion of ATP, through the process of oxidative phosphorylation. On the other hand, myofibrils by virtue of their calcium binding and ATP hydrolyzing abilities provide energy for contractile work. Thus any alteration in the functions of these cellular components can be conceived to modify the ability of myocardium to generate contractile force.

Over the past two decades, the actions of various interventions on myocardial contractility have been explained on the basis of their effects on one or more of the cellular organelles. Because of the lack of techniques available for monitoring the effects of different interventions on these cellular components in vivo, it is essential to study their interaction under in vitro conditions after separating these organelles into various fractions. In spite of the limitations of this approach, useful information on the mechanisms of action of different agents can be obtained by examining their dose - response relationships under a wide variety of experimental conditions.

Although various divalent cations such as $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ are known to depress myocardial contractility, their mode of action is far from clear. Electrophysiological, mechanical and calcium flux studies have indicated an impairment of the excitation - contraction coupling process by these heavy metals;

however, the exact site of their action is poorly understood. We believe that these cations act on myocardium through their interaction with different organelles, depending upon their concentration, and affect various sites involved in the regulation of calcium. Since no reports concerning the interaction of these divalent cations with myocardial organelles as a basis for their cardiopressant action has yet appeared in the literature, it was the purpose of this study to provide some information in this regard. It was designed to establish the subcellular basis of the cardiodepressant effect of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$.

In one series of experiments, the effects of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on the contractile force developement by the isolated perfused rabbit heart were investigated. An electron microscopic examination of these hearts was carried out to determine any changes in the myocardial ultrastructure due to these divalent cations. In another set of experiments, myofibrillar, mitochondrial, sarcoplasmic reticular and sarcolemmal fractions were isolated from unperfused rabbit hearts and the effects of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on various biochemical parameters of these fractions were studied. The ATP hydrolyzing abilities of these cellular fractions in the presence of $Ca^{++}$ and $Mg^{++}$ were measured. Furthermore, sarcolemmal calcium binding, adenylate cyclase and $Na^{+} - K^{+}$ ATPase activities were examined. The effects of these cations were also tested on the calcium accumulating abilities of mitochondrial and sarcoplasmic reticular fractions. In addition, mitochondrial oxidative phosphorylation activities were measured in the absence and presence of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$.

## II. REVIEW OF THE LITERATURE

### A. Regulation of Calcium Movements in Heart:

The importance of calcium in heart function has been recognized since Ringer (1) made the observation that heart was unable to contract when perfused with a medium lacking in calcium. Subsequently, it was shown that cessation of the mechanical activity of the heart on perfusion with a calcium - free medium was not associated with any change in the surface electrical activity (2, 3). By demonstrating that an intracellular injection of calcium is capable of initiating contraction, Heilbrunn and Wiercinski (4) further suggested that calcium is an essential link between excitation and contraction. Niedergerke (5, 6) showed that the calcium causing contraction exists in the ionized form and the force of contraction is related directly to the amount of ionized calcium in the cell. Since increasing the extracellular concentration of calcium during depolarization enhanced the mechanical activity of heart, it was claimed that calcium is the only ion present in body fluids which is capable of coupling excitation of the cell membrane to the contractile response of the cardiac muscle (7 - 9). These early electro-mechanical observations in addition to morphological and biochemical evidence (10 - 15) have established the fact that calcium is the final mediator in the excitation - contraction coupling process of the heart muscle. According to the current concept of cardiac excitation - contraction and relaxation processes (16 - 19) four cellular organelles namely sarcolemma, sarcotubular system, mitochondria and myofibrils are mainly involved in regulating calcium movements. Electrical depolarization initiated by the pacemaker is propagated along the sarcolemma and is believed to enter the cell through a transverse tubular system. This process is associated with an influx of calcium from the extracellular space and a release of calcium from superficial sites in the sarcolemma. In addition, there is a release of calcium from intracellular stores such as sarcoplasmic reticulum and possibly mitochondria through direct and indirect mechanisms. All these sources of calcium contribute in raising the concentration of free intracellular calcium from approximately $10^{-7}$ M to $10^{-6}$ - $10^{-5}$ M. This calcium binds to troponin and

relieves the inhibition exerted by troponin - tropomyosin system upon actin and myosin whereas hydrolysis of ATP due to stimulation of actomyosin ATPase provides energy for contraction of the myofibrils. The cytoplasmic concentration of free calcium is then lowered by a variety of mechanisms involving the sarcoplasmic reticulum, sarcolemma and possibly mitochondria and this process is believed to be associated with relaxation of myofibrils. Thus the cardiac contraction and relaxation cycle is generally viewed as the reflection of raising and lowering the intracellular concentration of free calcium. Furthermore, the molecular mechanisms of contraction and relaxation can be readily understood in terms of the functions of various membrane systems and contractile apparatus which intimately participate in regulating the movements of calcium in heart muscle. Although it is recognized that other cellular components such as the nucleus may also be involved in the regulation of intracellular calcium (20), no definitive information on this aspect is available in the literature at the present time.

Recently, extracellular calcium has been implicated in coupling excitation with contraction in cardiac muscle. Electrophysiological studies have provided evidence for a slow inward current of the cardiac action potential to be due to calcium (21 - 27). The magnitude of this current during the plateau phase of the action potential was relatively unaffected by external sodium ion concentration or tetrodotoxin, a specific antagonist of sodium influx. However, it was dependent upon external calcium concentration and was sensitive to calcium antagonists such as verapamil and its methoxy-derivative, D600 (28). Various cat ions such as $Ni^{++}$, $Co^{++}$, $Mn^{++}$ and $La^{+++}$ were found to depress contractile force by inhibiting the slow inward calcium current (29, 30). The threshold of the calcium current (about - 40 mV) must be reached before activation of the contractile apparatus takes place. Other voltage - clamp studies have failed to demonstrate a clear relationship between changes in the slow inward calcium current and contractile activity during the "staircase phenonemon" (23, 31, 32) or $Na^{+}$ - free perfusion (23, 31).

On the basis of total charge due to the calcium current it has been calculated that only 5 to 10 $\mu$ moles calcium influx/kg of heart weight occurs

during depolarization (24, 25). However, biochemical studies concerning measurements of myofibrillar calcium binding, ATPase activity and isometric tension at different concentrations of calcium revealed that about 85 μ moles of calcium/kg heart weight were necessary for full tension developement by the myocardium (33). From such experiments, it is clear that the calcium influx as measured by voltage - clamp techniques is not sufficient to fully activate the contractile apparatus upon depolarization. This point has been emphasized by Bassingthwaighte and Reuter (34) who presented evidence to support the view that additional calcium must be released from intracellular sites for full cardiac contraction. This, however, does not undermine the essential role played by extracellular calcium because $La^{+++}$, which does not penetrate the myocardial cell membrane, was found to uncouple excitation from contraction (35, 36).

The possibility that a small quantity of extracellular calcium entering during depolarization, as measured by voltage - clamp studies, could trigger the release of calcium from intracellular sites was suggested by some investigators (37, 38). This concept of "trigger calcium" was substantiated by Fabiato and Fabiato (39 - 41) when they showed that concentrations of calcium lower than that required for activating contraction of myofilaments directly were able to produce transient contractions in the cardiac cells with disrupted sarcolemma. The data from homogenized rat hearts (42) and chemically treated frog ventricles (43) have also been interpreted to support the hypothesis of regenerative calcium release from intracellular sites. However, it should be noted that calcium - induced calcium release can only be demonstrated clearly in preparations which are heavily loaded with calcium or treated with caffeine. Therefore, interpretation of results obtained from these unphysiological preparations should be taken with some caution.

Although release of calcium from the lateral cisternae of the sarcoplasmic reticulum directly by the wave of depolarization, travelling along the sarcolemma into the transverse tubular system, (44) can be conceived to occur in cardiac muscle, it should be noted that the sarcoplasmic reticulum in heart is not well developed (45, 46). Although Lee et. al. (47) demonstrated a release of calcium from the sarcoplasmic reticulum under the in vitro conditions, it is not clear

whether this effect was due to electrolysis or heat produced by the electric current. On the basis of the observation that changes in pH are associated with changes in calcium release from the sarcoplasmic reticulum (48) and the oscillation of intramuscular pH during contraction - relaxation cycle of cardiac muscle (49) it can be conceived that depolarization leads to changes in pH of the myoplasm and thereby causes a release of calcium from intracellular stores. Some investigators (50, 51) have suggested that release of calcium from intracellular stores is mediated by a rise in the intracellular sodium concentration whereas others (52) have attributed this event to an increase in the level of cyclic AMP. Irrespective of the mechanism of the release of calcium from the intracellular sites upon depolarization, the central role played by extracellular calcium in cardiac contraction can be readily appreciated by a rapid decline in contractile force upon perfusing hearts with calcium - free medium without any changes in the electrical activity (53).

Since myocardial cells such as the atrial and Purkinje cells (54), as well as the ventricular cells of embryonic or neonatal chickens have no transverse tubules (55), it appears that this membranous system is not essential for the rapid propagation of excitation throughout the cell or for the coupling of excitation to contraction. It should be noted that the time between excitation and the onset of contraction of cardiac muscle is about 20 m sec. Thus it is not essential that the wave of excitation release calcium from the sarcoplasmic reticulum almost simultaneously at the level of each sarcomere. On the other hand, current evidence points to the sarcolemmal basement membrane as the source of coupling calcium in the cardiac fiber (56). Accordingly, the calcium which is presented to the area of the contractile filament is released from the superficial cell membrane and travels inward to the area of the sarcomeres by a simple process of diffusion. Calcium ion diffuses in the myoplasm at a rate of 1 $\mu$/msec and would take 5 msec to reach the contractile unit of the cardiac cell with an average diameter of 10 $\mu$.

Although it is recognized that the superficial sites in heart sarcolemma are in equilibrium with calcium in the interstitial space, it is difficult to determine the amounts of calcium released from the superficial sites and entering from the extracellular space during depolarization. In this regard it should be noted that

the voltage - clamp studies do not detect the non - electrogenic movement of calcium into the myocardium as well as that released from the superficial sites in sarcolemma upon depolarization. Niedergerke (57) has already claimed that external calcium enters the myocardial cell through a carrier system. The existence of such a non - electrogenic carrier system, which moves calcium inward and sodium outward, has been shown by some investigators (58 - 60). A calcium - potassium exchange carrier has also been proposed for the myocardium (61). Thus, it appears that calcium enters the myocardial cell from the extracellular spaces and superficial sites in sarcolemma upon depolarization via electrogenic and non - electrogenic (carrier) mechanisms.

From the foregoing discussion, it is clear that different sources such as extracellular, sarcolemmal and intracellular sites participate in raising the myoplasmic level of free calcium upon depolarization of the cardiac cell. Data from calcium flux studies (13, 15, 62 - 68) have provided evidence regarding the existence of calcium in several compartments in cardiac muscle. It is generally believed that one compartment, which probably represents extracellular and sarcolemmal sources of calcium, is essential to the coupling process in cardiac muscle whereas the other compartment, which probably represents intracellular sources (sarcoplasmic reticulum and mitochondria), is intimately involved in the maintenance of contractile force (68, 69). Although these compartments have not been morphologically defined, some reports (70 - 72) indicate that certain interventions may modify the myocardial contractile force by influencing one or more of these calcium compartments. Because available techniques do not permit the exact localization of calcium compartments affected by various agents in the intact cardiac muscle, it is necessary to separate different cellular components into fractions and examine the effects of different interventions on their functions under in vitro conditions.

B. Interaction of Calcium with Cell Components:

Although considerable information is available on the electrical behaviour of the heart cell membrane, very little is known about its molecular composition and structure because of the difficulties involved in obtaining

sufficient material uncontaminated by intracellular organelles for biochemical analysis. The sarcolemma has two components, a thin electron dense plasma membrane and a much thicker, amorphous layer, basement membrane which coats the plasma membrane (73). The sarcolemma with its basement membrane invaginates at regular intervals into the myocardial cells forming the transverse tubular system which comes into close apposition with the lateral sacs of the sarcoplasmic reticulum, terminal cisternae. The basement membrane has been studied histochemically and has shown staining characteristics indicative of mucopolysaccharide or mucopolysaccharide - protein complex (74) with a high density of negatively charged sites, which are believed to selectively bind cations, notably calcium (75). There is a growing awareness that many cellular functions are directly controlled by macromolecules outside the cell, either as components of the plasma membrane or as cell - surface associated material (76). It is therefore possible that the basement membrane plays a crucial role in regulating myocardial function and metabolism. The importance of this layer in cardiac calcium movements has been recognized recently (15, 77) and it may well be that this site may be the source of "trigger" calcium. Whether or not this site is the same as the superficial calcium binding sites in sarcolemma is an open question at present. It also remains to be determined if the basement membrane plays any role as a calcium accumulating system outside the myocardial cell as proposed by Hajdu and Leonard (78) for maintaining a 3 - 4 fold higher calcium concentration in the microdomain of the cell in comparison to the extracellular space. Although it is conceivable that the basement membrane may be intimately involved in limiting calcium permeability across the plasma membrane, extensive research is required to gain insight into the functional aspect of this layer.

In contrast to the basement membrane, plasma membrane is considered to contain various enzyme systems which may be involved in the regulation of ionic permeability and modulation of myocardial contractility. Cytochemical evidence suggests the localization of both $Na^+ - K^+$ ATPase and adenylate cyclase in heart cell membrane (79, 80). The cardiac membrane fractions enriched in sarcolemma, obtained by employing different procedures, also showed high specific activities of

both $Na^+ - K^+$ ATPase and adenylate cyclase (81 - 86). The adenylate cyclase catalyzes the transformation of ATP into cyclic AMP which has been recognized as an important regulator of heart function and metabolism (87). On the other hand, $Na^+ - K^+$ ATPase has been shown to control myocardial function through the movements of sodium and potassium across the cell membrane (88). Although both enzymes are lipoproteins and require Mg ATP as a substrate, they differ in their responses towards cardioactive agents such as ouabain and epinephrine (89). For example, ouabain inhibited the sarcolemmal $Na^+ - K^+$ ATPase activity while epinephrine stimulated the sarcolemmal adenylate cyclase activity. The inhibition of $Na^+ - K^+$ ATPase by cardiac glycosides through a wide variety of mechanisms has been considered to augment calcium influx (18, 26, 88, 90). On the other hand, activation of adenylate cyclase is believed to be associated with an increase in calcium influx through the cyclic - AMP - protein kinase system (24, 91, 92). The role of $Na^+ - K^+$ ATPase in regulating calcium movements in heart under different experimental conditions has been emphasized (93), and dibutyryl cyclic AMP has been shown to enhance the uptake of calcium into the myocardium (91, 92). At any rate, both $Na^+ - K^+$ ATPase and adenylate cyclase in heart sarcolemma have been shown to be inhibited by calcium (89, 90, 94) but the exact significance of this effect is not clear at present.

The existance of an ATP - dependent "calcium pump," involved in lowering the intracellular concentration of free calcium in the myocardial cell during the relaxation phase, has been postulated in the heart sarcolemma (95). Although some investigators were able to show $Ca^{++}$ - stimulated $Mg^{++}$ dependent ATPase in heart sarcolemma (86, 96), the low enzyme activity reported by these investigators can be easily taken to be due to some technical artifact or contamination by the sarcoplasmic reticulum. The heart sarcolemmal preparation obtained by hypotonic shock - Li Br treatment was found not to exhibit $Ca^{++}$ - stimulated $Mg^{++}$ dependent ATPase (97). Furthermore, heart sarcolemma did not show ATP - dependent calcium binding (97, 98). It should be mentioned here that the inability to demonstrate a "calcium pump" in heart sarcolemma may be due to predominantly right - sided out orientation of the sarcolemmal membrane vesicles. Thus further

experiments are needed to provide convincing biochemical evidence concerning the presence or absence of a "calcium pump" mechanism in the heart sarcolemmal preparations. Electrophysiological and calcium efflux studies have indicated that extrusion of calcium from myocardium occurs through a carrier involving the $Na^{+}$ - $Ca^{++}$ exchange mechanism (26, 99). Although ATP dependent calcium binding by heart sarcolemma has been described recently (86), the values reported are rather low. The heart sarcolemmal preparations, however, have been shown to contain $Ca^{++}$ - ATPase and $Mg^{++}$ - ATPase activities (81, 84, 85, 98, 100). Although both these enzymes have been suggested to be involved in the movements of divalent cations across the cell membrane (97, 100), the exact manner of their participation in the movements of cations is far from clear. In addition, it is not known whether the ATP hydrolysis in the presence of $Ca^{++}$ or $Mg^{++}$ is due to the presence of the same or different enzyme complex in heart sarcolemma.

The identification of a "calcium pump" mechanism in the sarcoplasmic reticular system as the cause of relaxation appears to have been well documented and this organelle is envisioned to release calcium upon excitation of the cardiac cell (11, 12, 101 - 105). Various investigators have attempted to improve the calcium accumulating activity of heart sarcoplasmic reticulum by isolating and incubating these fragmented vesicles under different experimental conditions (106 - 114). ATP - dependent calcium accumulation by the fragments of sarcoplasmic reticulum (microsomal fraction) is generally studied in the presence of a permeant anion, oxalate, and this process is termed as calcium uptake. On the other hand, ATP - dependent calcium accumulation in the absence of permeant anion is called calcium binding. Although it is recognized that calcium binding represents an arbitrary meaning of the term because some calcium uptake may occur within the reticular vesicles under the experimental conditions usually employed for studying this process, it should be noted that in contrast to calcium uptake activity, the maximal calcium binding activity is observed within 2 to 5 minutes. Furthermore, the range of maximal calcium binding activities for the cardiac microsomal fractions from different species is 25 to 60 n moles $Ca^{++}$/mg protein whereas the values for calcium uptake activities vary between 700 - 3,000 n moles $Ca^{++}$/mg protein.

Species dependent differences, both in calcium binding and uptake activities of the heart microsomal fractions have recently been emphasized (115, 116).

Although there is some controversy concerning the usuage of the terms calcium binding and calcium uptake, there is evidence that these may be two different processes. In this regard it has been shown that the microsomal calcium uptake, but not calcium binding, was increased by cyclic AMP - protein kinase (117, 118). Different agents such as antibiotic ionophore, X 537A, and arsenate were reported to be more potent inhibitors of calcium uptake than of calcium binding by heart microsomes (119). Furthermore, depression of calcium uptake and stimulation or no effect on calcium binding by heart microsomes were shown to occur in the presence of quinidine (120, 121). Calcium binding by the fragments of sarcoplasmic reticulum from different types of failing hearts was observed to be depressed under experimental conditions, showing no alteration in calcium uptake (122 - 125). It is possible that both calcium binding and uptake processes may share the same sites, at least during the initial phase, but convincing evidence in support of this view is lacking. Furthermore, the exact role played by these two processes in myocardial function is far from clear.

Scattered and conflicting information regarding the influence of different ions on calcium uptake by the microsomal fraction is available in the literature. For example, Carsten (126) has reported that the rate of calcium uptake in the sarcoplasmic reticulum of the dog heart is neither affected by changes in $K^+$ or $Na^+$ concentrations nor by substitution of isosmolar amounts of sucrose for 70% of the $Na^+$. On the other hand, Katz and Repke (127) have demonstrated that concentrations of KCl or Na Cl below 0.1 M increased calcium uptake by the dog heart sarcoplasmic reticulum. Furthermore, replacement of KCl by equimolar amounts of Na Cl decreased both the rate and extent of calcium uptake by the microsomal fraction. Palmer and Posey (128) have provided evidence that the reduction of calcium uptake by $Na^+$ is due to a rapid release of calcium bound to the microsomal membrane. These studies have not been carried out in detail under identical experimental conditions nor do these reports provide enough experimental basis for the localization of the site for intracellular ionic competition which appears to be

rather critical for determining the regulation of myocardial contractility. Although some data concerning calcium release from the microsomal fraction is also available in the literature (48, 119, 129), the results are too preliminary to warrant further comments.

It is pertinent to mention that heart mitochondria, like the fragments of sarcoplasmic reticulum, have been shown to accumulate calcium (130 - 134). From time to time, various investigators have expressed their concern that mitochondria are involved in the regulation of intracellular concentration of calcium (135 - 140). It was interesting to observe that these organelles contained the highest specific activity upon exposure of the whole heart, both under in vitro and in vivo conditions, to radioactive calcium (20, 140, 141). The energy dependent calcium accumulation in mitochondria has been shown to occur in vitro by either respiration supported or ATP - dependent processes. However, under in vivo conditions, mitochondria can be conceived to accumulate calcium by both ATP and respiration dependent mechanisms but the extent of their contribution in this process is not known. It should be pointed out that calcium in high concentration has been shown to depress the mitochondrial respiratory and oxidative phosphorylation activities (133). At any rate, respiration - linked calcium transport by mitochondria has been reported to occur in preference to ATP formation (142).

Energy dependent calcium binding by heart mitochondria is usually studied in the absence of a permeant anion such as phosphate; however the occurrance of some calcium accumulation within mitochondria under this condition can not be ruled out. On the other hand, calcium uptake by mitochondria is measured in the presence of inorganic phosphate. The ranges for heart mitochondrial calcium binding and calcium uptake activities in different species have been observed to be 30 - 70 and 200 - 500 n moles $Ca^{++}$/mg protein/5 min respectively. While the process of calcium transport in mitochondria has been recognized to be of a complex nature (143 - 147), Schuster and Olson (148) have suggested it to consist of at least 3 phases: (a) an energy - independent binding of calcium to the surface of the membrane, (b) an energy - dependent movement of calcium in or on the membrane, and (c) an anion - dependent transfer of calcium from the membrane into the

matrix space.

In spite of the fact that both sarcoplasmic reticulum and mitochondria can accumulate calcium in an energy dependent manner, a great many differences between these cellular structures have been observed with respect to their calcium transport systems. For example, $Ca^{++}$ - stimulated $Mg^{++}$ dependent ATPase, the enzyme which is intimately involved in calcium transport, can be easily demonstrated by employing the fragments of sarcoplasmic reticulum but not with mitochondria. The uptake of calcium by heart mitochondria is inhibited by oligomycin, azide, dicumarol and dinitrophenol whereas these agents do not have significant effects on the microsomal calcium uptake (11, 20, 149, 150). Unlike the cardiac microsomal fraction, the calcium binding by mitochondria is decreased in the presence of 5' - AMP, 3' - AMP and 5' - IMP. Furthermore, both 3' - AMP and adenosine have been found to inhibit the ATPase activity of mitochondria without affecting the microsomal enzyme (151). Quinidine was also found to have different actions on calcium binding by heart mitochondrial and microsomal fractions (120). Although the cardiac microsomal fraction has been shown to contain more neutral lipids and phospholipids in comparison to the mitochondrial fraction (152, 153), a satisfactory explanation for differences in the mechanism of calcium transport by mitochondria and sarcoplasmic reticulum must await further investigations. At any rate, a similar structural role of phospholipids in mitochondrial and sarcoplasmic reticular membranes has been clearly demonstrated (154).

The participation of mitochondria in excitation - contraction coupling is generally questioned on the basis of lack of continuity of these organelles with the cell membrane or transverse tubular system. However, such a problem does not arise if one assumes that calcium release from mitochondria is mediated by a chemical stimulus rather than via an electrical event. In this regard it should be noted that efflux of calcium from mitochondria has been shown to be accompanied by uptake of $H^{+}$ from the suspending medium just as calcium uptake is accompanied by $H^{+}$ ejection (155). Haugaard et. al. (136) have considered that the relative concentrations of ATP, Pi and $Mg^{++}$ are important in determining the direction of calcium movements in mitochondria. Furthermore, glycolytic intermediates, such as phos-

phoenol - pyruvate, have been shown to increase the rate of efflux of mitochondrial calcium (156). Several agents such as uncouplers of oxidative phosphorylation and respiratory inhibitors can induce calcium release from mitochondria but the interpretation of these results in terms of myocardial function is rather difficult. $Na^+$ has also been shown to release mitochondrial calcium (50, 157) but whether this occurs under physiological conditions is a matter of speculation. It should be noted that mitochondria have been observed to inhibit myofibrillar syneresis and ATPase activity (158, 159), and remove bound calcium from troponin (160). Although Carafoli (161) has emphasized the role of mitochondrial calcium during the cardiac contraction - relaxation cycle, other workers (162, 163, 164) are strongly opposed to this view. From the foregoing discussion it is clear that both sarcoplasmic reticulum and mitochondria are capable of regulating the intracellular concentration of calcium. It should, be noted that the rate and extent of calcium uptake by heart mitochondria are considered to be slower than those by the microsomal fraction (20, 106, 135, 165), however, none of the investigators have employed identical conditions for such studies with subcellular fractions. The Km values for calcium transport by microsomal and mitochondrial fractions were reported to be 1.6 and 12.1 μM respectively (164). On the other hand, Scarpa and Graziotti (163) have found the Km values for heart mitochondrial calcium transport in different species to vary between 30 - 92 μM whereas Carafoli (161) has arrived at a value in the vicinity of 1 μM. Such differences in results are most likely due to the experimental conditions employed for the isolation of subcellular membranes as well as the techniques used for evaluating the calcium transport ability. Thus the contribution of mitochondria and sarcoplasmic reticulum in regulating intracellular concentration of calcium during contraction - relaxation processes in the heart remains controversial, though most of the investigators agree that mitochondria are involved in regulating intracellular calcium and may serve as a "calcium sink."

The interaction of calcium with cardiac contractile proteins has recently been reviewed (19, 166, 167). It is becoming clear that the contractile apparatus is made up of thick (myosin) and thin (actin) filaments which slide over each other during contraction and proteins such as troponin and tropomyosin regulate their

movements. Myosin possesses ATPase activity and troponin serves as a receptor for calcium. The level of calcium which produces half maximal myofibrillar ATPase activity has been found to be the same for that producing half maximal tension developement (43). However, a clear relationship between myofibrillar ATPase and calcium binding was not observed, due to the presence of more than one calcium binding site (165).

Troponin has been shown to bind calcium at two different sites and the Km for the high affinity site is about 2 μM (12, 168). Likewise, the Km for actomyosin ATPase was also found to be 2 μM of $Ca^{++}$ (165). While it is believed that calcium by binding to troponin relieves the inhibitory effect of troponin - tropomyosin on the actin and myosin filaments, it is not clear whether the activation of myofibrillar ATPase by calcium occurs through a similar mechanism or is a result of a direct action of calcium on myosin ATPase. It should also be mentioned that in addition to the presence of $Ca^{++}$ - stimulated $Mg^{++}$ dependent ATPase activity, myofibrils are known to possess $Mg^{++}$ ATPase (basal ATPase) activity. The role of $Mg^{++}$ ATPase, however, has not been defined with respect to the functional aspect of the contractile proteins. It is now well established that ATP is the most immediate source of energy utilization by myofibrils for contraction and the binding of calcium to troponin regulates tension developement.

C. Pharmacologic Interventions and Cellular Components

A number of pharmacologically active agents have been considered to influence myocardial function through their effects on one or more cellular organelles such as sarcolemma, mitochondria, sarcoplasmic reticulum and myofibrils. Such mechanisms of drug action are usually based on observations obtained from in vitro experiments by studying the activities of various cellular fractions in the presence of different doses of a given drug. Although it is possible that the sensitivities of the cellular fractions may change during the experimental conditions employed for isolation and incubation, most investigators agree that some valuable information can be derived from such an approach. For example, drugs may produce positive or negative inotropic effects by increasing or decreasing the myofibrillar ATPase activity respectively. Different agents may impair myocardial contractility by

decreasing calcium transport by mitochondria and/or sarcoplasmic reticulum. Furthermore, some agents may influence myocardial function by altering calcium movements through their actions on sarcolemmal calcium binding, $Na^{+} - K^{+}$ ATPase, $Ca^{++}/Mg^{++}$ ATPase and adenylate cyclase. In general, different agents may be considered to have one or more sites of action on cellular components.

It has been known for a long time that cardiac glycosides produce a positive inotropic effect in heart; however, conflicting reports concerning their mode of action have appeared in the literature (88, 90, 169). Many investigators have reported that ouabain and strophanthidin depress calcium uptake by mitochondria and sarcoplasmic reticulum (126, 159, 170, 171) whereas others have denied such actions (117, 135, 172, 173). Cardiac glycosides do not influence myofibrils but they have been shown to inhibit sarcolemmal $Na^{+} - K^{+}$ ATPase (85, 89, 90). The inhibition of heart sarcolemmal $Na^{+} - K^{+}$ ATPase by cardiac glycosides is considered to increase the contractile force by direct or indirect mechanisms, though, it should be pointed out that the inhibition of $Na^{+} - K^{+}$ ATPase does not appear to be a universal requirement for the production of a positive inotropic effect by other agents.

The mechanism of action of catecholamines, glucagon or cyclic AMP on calcium transport by the microsomal fraction has received much attention in order to explain the positive inotropic action of these agents. Some investigators reported a direct action of catecholamines, glucagon or cyclic AMP on microsomal calcium uptake (174 - 177) whereas others have failed to confirm such a finding (117, 172, 178 - 183). These conflicting reports may be due to the presence of varying amounts of protein kinase and adenylate cyclase activities (184 - 186) in the microsomal preparations. Stimulation of heart microsomal calcium uptake by cyclic AMP - dependent protein kinase has now been reported from different laboratories (187 - 189). This enhancement of microsomal calcium uptake and $Ca^{++}$ - stimulated ATPase activities by cyclic AMP - dependent protein kinase seems to be due to phosphorylation of the microsomal membranes (190 - 192). The significance of this mechanism under in vivo situation has now been clearly demonstrated (193).

In addition to increasing calcium uptake by the microsomal fraction,

cyclic AMP - dependent protein kinase has also been shown to increase ATP - dependent calcium binding by heart sarcolemma (86, 194). Although increase in calcium uptake by microsomal fraction has been suggested to shorten the relaxation phase of myocardium, it may well be that cyclic AMP - dependent protein kinase increases the calcium content of the sarcoplasmic reticulum and allows more calcium to be available for the contractile apparatus during depolarization. An increase in the sarcolemmal "calcium pump" activity due to cyclic AMP - dependent protein kinase will also contribute in reducing the time for relaxation. Cyclic AMP - dependent protein kinase has also been reported to phosphorylate troponin and this action has been suggested to modulate cardiac contraction and relaxation (195). However, it should be noted that microsomal calcium binding and mitochondrial calcium transport activities were not affected by cyclic AMP - dependent protein kinase (117, 118). Thus it appears that agents which increase cyclic AMP levels may influence myocardial contractility at least in part through protein kinase dependent phosphorylation of sarcolemma, sarcoplasmic reticulum and troponin - tropomyosin system. Although these agents are also known to increase calcium influx through myocardial sarcolemma, it is not clear whether this effect is mediated through the cyclic AMP - protein kinase system or is associated with some separate mechanism.

Various investigators have reported the effects of some well known $\beta$ - adrenergic blocking drugs on heart membranes. For example, propranolol was shown to inhibit calcium uptake and ATPase activities of the microsomal fraction (196 - 201). This inhibitory effect of propranolol was reversed by catecholamines (197, 198) while other investigators (200, 201) have failed to confirm this finding. Propranolol was also found to inhibit calcium uptake by mitochondria (201 - 203) and this effect was not antagonized by catecholamines (201). Other agents such as acebutolol and practolol, which are more cardio-selective with respect to their $\beta$ - adrenergic receptor blocking activity in comparison to propranolol, were markedly less potent in depressing calcium uptake by subcellular fractions as well as in producing cardiodepressant effects (201). The inhibitory effect of propranolol on the microsomal membranes was found to be associated with a depression of the

sarcotubular - $\gamma$ - $AT^{32}P$ reaction (204). No convincing evidence concerning the effects of propranolol on heart sarcolemma and myofibrils has yet appeared in the literature. Therefore, we believe that the inhibition of sarcoplasmic reticular and mitochondrial calcium transport represents the biochemical mechanism of the cardiodepressent effect of propranolol which does not appear to be associated with its $\beta$ - adrenergic blocking activity.

Quinidine and other antiarrhythmic agents, in high doses, are known to depress the ability of heart to generate contractile force. This pharmacological effect of quinidine has been explained on the basis of its inhibitory effect on the calcium uptake activity of the microsomal fraction (120, 205 - 208). It should be noted that quinidine was reported to decrease microsomal calcium uptake due to its inhibitory effect on the sarcotubular - $\gamma$ - $AT^{32}P$ reaction (209). Heart mitochondrial calcium uptake was also decreased by quinidine but other antiarrhythmic drugs such as procaine amide and lidocaine had no effect on microsomal or mitochondrial calcium uptake (120, 208). Myofibrillar ATPase activities were not altered by these antiarrhythmic agents which were found to change some of the sarcolemmal bound enzyme activities (208). Quinidine, but not procaine amide or lidocaine, was observed to depress mitochondrial oxidative phosphorylation activities. In addition quinidine and lidocaine were reported to decrease the rate of transmembrane $Ca^{++}$ exchange during excitation of myocardium (210). It seems likely that quinidine may exert its cardiodepressant effect by acting on several sites such as sarcolemma, mitochondria and sarcoplasmic reticulum whereas both procaine amide and lidocaine may produce negative inotropic effects by acting on sarcolemma.

The barbiturates have been found to depress calcium uptake by sarcoplasmic reticulum and mitochondria. Lain et. al. (211) and Nayler and Szeto (212) have reported that millimolar concentrations of sodium pentobarbital decreased the ability of the cardiac microsomal fraction to accumulate calcium. Briggs et. al. (213) also found that amytal inhibited calcium uptake by the microsomes and this action was found to be due to an interference with calcium binding to the microsomal phospholipids (214). On the other hand, Dransfield et. al. (215) failed to detect

any effect of 1 - 6 mM pentobarbital on calcium uptake by microsomal fraction but have reported its inhibitory action on mitochondrial calcium uptake. Although other anesthetic agents such as chloroform and halothane depressed microsomal calcium transport, ether was found to show no such effect (211). Both calcium binding and calcium uptake by cardiac microsomes were also inhibited by ethanol (216) whereas hexobarbitone has been shown to decrease transmembrane calcium exchange during excitation of the myocardium (217). It is therefore likely that different anesthetic agents may depress calcium movements at sarcolemmal, mitochondrial and/or microsomal membrane and thus produce cardiac depression. It is, however, recognized that a great deal of work concerning the subcellular effects of anesthetic agents remains to be done before any definitive conclusion can be reached regarding their exact sites of action.

Several calcium antagonistic agents such as verapamil and its methoxy derivative, D600, are believed to produce negative inotropic effects by blocking specific calcium channels in the cell membrane (28, 218, 219). However, verapamil has recently been reported to be ineffective in reducing calcium influx during excitation of myocardium (220). On the other hand, Nayler and Szeto (21) have found that verapamil impaired the capacity of the sarcolemmal binding sites to accumulate calcium while having no action on the ability of the microsomal fraction to accumulate, bind and exchange calcium. Entman et. al. (129) and Watanabe and Besch (222) have observed that verapamil in high concentrations was capable of decreasing microsomal calcium binding and uptake activities. In contrast to these agents, ryanodine, has been suggested to decrease myocardial contractility by decreasing the microsomal calcium uptake activity (223, 224). On the other hand, pyridine aldoxine methochloride has been reported to decrease calcium uptake by the microsomal fraction which action has been suggested to be responsible for its positive inotropic effect (225). It should be noted that some agents such as caffeine and antibiotic ionophores have been found to produce positive inotropic effect by releasing calcium from mitochondria and sarcoplasmic reticulum respectively (226 - 228). These studies clearly reflect the complexities involved in determining the site of drug action as well as interpretation of the data obtained from

in vitro experiments.

From the foregoing discussion it appears that various cardioactive agents may modify the contractility of the heart through their effects on calcium movements by sarcolemma, sarcoplasmic reticulum and mitochondria whereas myofibrils seem to be more resistant to the drug action. The information concerning the actions of different pharmacological agents on heart sarcolemma and mitochondria is sparse in comparison to that available for the sarcoplasmic reticulum. Most of the studies with sarcoplasmic reticular calcium uptake have been carried out in the presence of oxalate which makes the results difficult to interpret because of its unphysiological nature whereas, very little attention has been paid to studying the drug action on calcium binding, calcium release and calcium uptake in the presence of phosphate. In spite of the lack of such information, it is currently believed that drugs decrease calcium uptake by sarcoplasmic reticulum and/or mitochondria reducing the intracellular calcium stores and thus, less calcium is available for release upon excitation of the myocardium. In addition, pharmacologic agents may also produce a negative inotropic action by decreasing calcium bound to superficial sites on sarcolemma or reducing transmembrane calcium influx. On the other hand, drugs may increase myocardial contractility by making more calcium available for release from the intracellular and sarcolemmal sites or by increasing calcium influx from the extracellular space. No conclusive evidence concerning changes in calcium release from the external or internal calcium stores is available in the literature because of the difficulties in studying this phenomenon under in vitro conditions. The exact manner by which changes in sarcolemmal enzymes due to different agents affect calcium movements across the cell membrane is also not clear at present. Thus a great deal of work needs to be carried out to understand the subcellular and molecular basis of drug action.

D. Effects of Some Divalent Cations on Myocardial Function

In contrast to $Ca^{++}$, various divalent cations such as $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ are known to depress myocardial contractility by interfering with the excitation-contraction coupling mechanism (24, 28, 29). Both $Co^{++}$ and $Ni^{++}$ were reported to decrease contractile force without affecting the height and the shape of the action

potential (229). Furthermore, these divalent cations were found to selectively depress transmembrane calcium current which was restored to a varying degree by increasing the extracellular concentration of calcium (230, 231). From these studies it appears that $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ act on the cell membrane and prevent calcium influx by some competitive mechanism. However, other investigators have shown that $Mn^{++}$ produced a marked decrease in the plateau of the action potential (232, 233). Complex effects of $Mn^{++}$ on cardiac membrane potentials have also been described and this ion under certain conditions has been shown to generate a calcium - like transmembrane current in myocardial cells (234 - 236).

In addition to depressing myocardial contractility, $Mn^{++}$ has been shown to produce contracture and accumulate within the cardiac cell (234). From these observations it was suggested that $Mn^{++}$ interferes with excitation - contraction coupling not only at the sarcolemmal level but also at some intracellular sites. Calcium exchange studies have revealed that $Mn^{++}$ may reduce calcium influx by displacing $Ca^{++}$ from superficial sites on the sarcolemma (237, 238). On the other hand, the ability of $Mn^{++}$ to antagonize the action of digitalis glycosides on contractile force (239) and automaticity (240) has been interpreted to be due to its effects at the membrane and cellular storage sites and therefore influence calcium movements (239). $Ni^{++}$ has been suggested to compete for some intracellular sites after the release of calcium from the pool required for the maintenance of contractile force (241). Thus it appears that the exact mechanism by which these divalent cations impair the excitation - contraction coupling process is far from clear.

Unlike calcium, both $Ni^{++}$ and $Co^{++}$ failed to restore the ability of heart to develop contractile force after perfusion with $Ca^{++}$ - free medium (242). Furthermore, these divalent cations did not release intracellularly bound $Ca^{++}$ from the cardiac cells but instead were shown to release calcium from the superficially located pool (242). Thus it is possible that different divalent cations may decrease the calcium content at the superficial sites of sarcolemma and make less calcium available upon excitation. The depressant effect of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ has also been explained on the basis of their interference with the release of calcium from the storage sites (243).

The toxic action of heavy metals including $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on myocardium has been known for a long time (244). Cobalt has been shown to produce myocardial cell damage in different experimental models (245 - 250). Diverse clinical aspects of cobalt induced cardiomyopathy have been described in detail (251 - 255). Dramatic hemodynamic, electrocardiographic, pathological and biochemical changes have been shown to be associated with cobalt-induced cardiomyopathy (256 - 261). Chronic doses of cobalt given to animals have also been reported to produce alterations in myocardial energy metabolism (262, 263). However, it is pointed out that very little is known about ability of both $Mn^{++}$ and $Ni^{++}$ to produce myocardial cell damage and changes in myocardial metabolism; however, such effects are conceivable in view of their similarities with $Co^{++}$ in their mode of action.

From the foregoing discussion it is apparent that different divalent cations notably $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, are capable of affecting myocardium; however the exact mechanism of their cardiodepressant action is poorly understood. Electrophysiological and mechanical studies have indicated an impairment of the excitation - contraction coupling mechanism. In this regard, selective inhibition of calcium influx, displacement of superficially - bound calcium, impairment of calcium release from storage sites and interference with processes after the release of calcium from storage sites have been suggested as possible mechanisms for their cardiodepressant effects. On the basis of their interaction with some cardioactive agents as well as calcium flux studies the possibility that these cations may compete with calcium at troponin (264) and myosin (265) can not be ruled out at present. Furthermore, it can not be stated with certainity whether or not the cardiodepressant effect of these divalent ions are associated with different enzyme systems involved in metabolic regulation (266 - 270). It should be noted that virtually nothing is known concerning the effects of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on functions of various myocardial cell components such as sarcolemma mitochondria, sarcoplasmic reticulum and myofibrils.

## III. METHODS

Healthy, male New Zealand White rabbits (3 to 5 kg each) were used in this study. All the animals were kept in environmentally controlled rooms, maintained on a standard rabbit diet fed ad libitum and had free access to water.

### A. Isolated Heart Preparation

Rabbits were sacrificed by cervical dislocation, the hearts quickly removed and chilled in ice - cold perfusion medium. The atria, fatty material and connective tissue were trimmed and the hearts were arranged for coronary perfusion by the Langendorff technique as described previously (271). Hearts were equilibrated for 15 min with Krebs - Henseleit solution of the following composition (mM): Na Cl, 120; Na $HCO_3$, 25; KCl, 4.8; $KH_2PO_4$, 1.25; $MgSO_4$, 1.25; $CaCl_2$, 1.25; and glucose, 8.6. The perfusion medium, pH 7.4, was oxygenated with a gas mixture of 95% $O_2$ and 5% $CO_2$ and maintained at a temperature of 37°C. The hearts were stimulated at 240 beats/min with a square-wave pulse just above the threshold and the coronary flow rate was maintained at 25 ml/min. A resting tension of 5 gm was applied to the heart at the beginning of the experiment. The contractile force (developed tension) and the rate of change of force developement (df/dt) were monitored on a Grass polygraph recorder with a force displacement transducer (FT.03). For studying the effects of different divalent cations, the hearts were switched to perfusion media containing different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$.

### B. Electron Microscopic Examination

At the end of perfusion of the hearts with medium in the absence or presence of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, the hearts were abruptly switched to perfusion with a solution of 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 min. Portions of papillary muscles and biopsies of the left ventricle tissue were further fixed in 1% glutaraldehyde solution for 1 to 2 hr. The tissue specimens were washed overnight in 0.1 M phosphate buffer, fixed for 1 hr with 1% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Epon 812 according to the method of Luft (272). The sections obtained with the use of a Blum MT - II ultramicrotome and glass knives, were stained with uranyl acetate and lead citrate and

examined with a Zeiss electron microscope (EM 9S).

C. Isolation of Cellular Components

Rabbits were sacrificed by cervical dislocation, the hearts were quickly removed and the ventricles dissected out and employed for isolation of various cellular components. All isolation procedures were done in a cold room at 0 to 4° C.

(1) Isolation of the myofibrillar fraction

Procedure A for isolating myofibrils is the same as described by Muir et. al. (273). Hearts were washed and homogenized in 5 volumes of 0.1 M KCl, 5 mM Tris - HCl, pH 7.0 and centrifuged at 5,000 x g for 10 min. This procedure was repeated twice and the pellet was suspended in 5 volumes of 40% sucrose, 0.1 M KCl, 5 mM Tris - HCl, pH 7.0 and centrifuged at 15,000 x g for 10 min. The residue after two washings was centrifuged at 5,000 x g for 10 min and suspended in 0.1 M KCl, 5 mM Tris - HCl, pH 7.0. This preparation was employed only in some experiments for the purpose of comparison.

Procedure B for the isolation of myofibrils is the same as that described by Solaro et. al. (274). Hearts were washed with ice - cold 0.9% NaCl. Fat and connective tissue were trimmed away and the ventricles were homogenized in 4 volumes of 0.3 M sucrose containing 10 mM imidazole, pH 7.0 for 1 min. The homogenate was centrifuged at 17,300 x g for 20 min and the pellet was suspended to the original homogenate volume in 60 mM KCl, 30 mM imidazole, 2 mM $MgCl_2$, pH 7.0. The suspension was centrifuged at 750 x g for 15 min and this sequence of centrifugation and resuspension was repeated 4 times. The pellet obtained after these washes was suspended in 60 mM KCl, 30 mM imidazole, 2 mM $MgCl_2$, pH 7.0, containing 2 mM EGTA (ethyleneglycol-bis ( $\beta$ - aminoethylether) - N, N' - tetraacetic acid) and centrifuged at 750 x g for 15 min. Further purification of the myofibrils was achieved by resuspending the EGTA treated, washed myofibrils in 8 pellet volumes of 60 mM KCl, 30 mM imidazole, 2 mM $MgCl_2$, pH 7.0 containing 1% Triton X - 100 and centrifuged at 750 x g for 15 min. The Triton X - 100 treatment was repeated once. The resulting pellet was washed 4 times to remove the Triton X - 100 and suspended in 60 mM KCl, 30 mM imidazole, pH 7.0.

(2) Isolation of heavy microsomal fraction

Hearts were thoroughly washed with 0.25 M sucrose containing 1 mM EDTA (ethylenediaminetetraacetate), pH 7.0. The tissue was homogenized in 10 volumes of 10 mM sodium bicarbonate, 5 mM sodium azide and 15 mM Tris - HCl, pH 6.8 in a Waring blendor for 45 sec. The homogenate was filtered through four layers of gauze and centrifuged at 10,000 x g for 20 min to remove cell debris, nuclei, myofibrils and mitochondria. The residue was discarded and the supernatant was centrifuged at 40,000 x g for 45 min. The sediment after this centrifugation 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 min. This procedure was repeated twice and the final pellet was suspended in 50 mM KCl, 20 mM Tris - HCl, pH 6.8. This method of isolation of the heavy microsomes is essentially similar to that described by Harigaya and Schwartz (106).

(3) Isolation of mitochondrial fraction

Hearts were thoroughly washed in 0.25 M sucrose, containing 1 mM EDTA, pH 7.0. The tissue was homogenized in 10 volumes of 0.18 M KCl, 10 mM EDTA, 0.5% albumin (fatty acid free), pH 7.4 in a Waring blendor for 20 sec. The homogenate after filtering through two layers of gauze was centrifuged at 1,000 x g for 20 min to remove cell debris, nuclei and myofibrils. The supernatant was centrifuged at 10,000 x g for 20 min and the resulting pellet was suspended in the homogenizing medium and centrifuged at 1,000 x g for 10 min. The supernatant was further centrifuged at 8,000 x g for 10 min. This washing procedure was repeated twice and the final pellet was suspended in 50 mM KCl, 20 mM Tris - HCl, pH 6.8. This procedure for isolating the mitochondrial fraction is similar to that described by Sordahl and Schwartz (275).

(4) Isolation of sarcolemmal fraction

Hearts were washed in ice - cold 10 mM Tris - HCl, pH 7.4 and homogenized with 10 volumes of 10 mM Tris - HCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.4 in a Waring blendor for 1 min (30 sec x 2, with an interval of 1 min). The homogenate was filtered through 2 layers of gauze and centrifuged at 1,000 x g for 10 min. The sediment was suspended in the above buffer, stirred in a cold room

for 15 min and centrifuged at 1,000 x g for 10 min. The sediment was suspended in 10 mM Tris - HCl, 1 mM DTT, pH 8.0, stirred for 15 min and centrifuged at 1,000 x g for 10 min. The sediment was suspended in 10 mM Tris - HCl, 1 mM DTT, pH 7.4, stirred for 15 min and centrifuged at 1,000 x g for 10 min; this step was then repeated again. The sediment was suspended in 10 mM Tris - HCl, 1 mM DTT, pH 7.4 and extracted with 0.4 M Li Br for 30 min and centrifuged at 1,000 x g for 10 min. The sediment after washing with the above Tris - buffer was further extracted with 0.6 M KCl, 1 mM DTT for 15 min, and centrifuged at 1,000 x g for 10 min. The sediment was washed with 10 mM Tris - HCl, pH 7.4 and suspended in 1 mM Tris - HCl, pH 7.4 and employed for biochemical studies. This procedure is essentially similar to that described by McNamara et. al. (85).

D. Biochemical Studies

All the cellular fractions employed in this study were used within 1 - 2 hr of their isolation. These cellular fractions were routinely checked for purity by measuring marker enzyme activities and electron microscopic examination (85, 124) and only minimal (4 - 6%) cross contamination was noted.

(1) Determination of calcium accumulating activities

Calcium binding by mitochondrial, microsomal and sarcolemmal fractions was determined by employing the millipore filtration technique (124). For microsomal and mitochondrial calcium binding, the fractions (0.2 to 0.3 mg/ml) were preincubated in medium containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris - HCl, pH 6.8, 4 mM ATP in a total volume of 1 ml. The reaction at 25°C was started by the addition of 0.1 mM $^{45}CaCl_2$, allowed to proceed for a further 5 min and stopped by millipore filtration (pore size 0.45 μ). Calcium binding by the sarcolemmal fraction was determined in a medium containing 50 mM Tris - HCl, pH 7.0 at 37°C with a protein concentration of 0.15 to 0.20 mg/ml. The reaction was initiated by the addition of either 0.1 mM of 1.25 mM $^{45}CaCl_2$ and terminated by millipore filtration. All the fractions were preincubated for 3 min in the absence or presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The radioactivity in the protein - free filtrates were estimated in a Packard Liquid Scintillation Spectrometer using Bray's solution.

Calcium uptake by the microsomal fraction (0.02 to 0.04 mg/ml) was determined in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris - HCl, pH 6.8, 5 mM potassium oxalate, 4 mM ATP in a total volume of 1 to 3 ml. Mitochondrial fraction (0.1 to 0.2 mg/ml) was incubated in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris - HCl, pH 6.8, 4 mM $KH_2PO_4$, 4 mM sodium succinate, and 4 mM ATP in a total volume of 1 to 3 ml. In some experiments calcium uptake by mitochondria was determined in the presence of pyruvate (1.5 mM) - malate (0.5 mM) or 4 mM ATP. The fractions were preincubated for 3 min in the absence or presence of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ and the reactions at 37°C were started by the addition of 0.1 mM $^{45}CaCl_2$, allowed to proceed for 5 min and terminated by millipore filtration.

(2) Determination of ATP hydrolyzing abilities

The ATPase activity of the mitochondrial fraction was determined at 37°C in a medium containing 100 mM KCl, 20 mM Tris - HCl, pH 6.8, 10 mM $MgCl_2$, and 4 mM ATP whereas that of the microsomal fraction was measured at 37° C in a medium containing 100 mM KCl, 20 mM Tris - HCl, 10 mM $MgCl_2$, pH 6.8, 4 mM ATP, 5 mM potassium oxalate and either 0.1 mM $CaCl_2$ or 1 mM EGTA. For the microsomal fraction the value in the presence of EGTA is taken as basal activity whereas the difference between the basal and total (in the presence of 0.1 mM $CaCl_2$) activities was considered due to $Ca^{++}$ - stimulated ATPase. The concentrations of the mitochondrial and microsomal proteins employed here were the same as those for the calcium uptake studies.

The ATPase activity of myofibrils obtained by Procedure A was determined in the absence and presence of 5 mM sodium azide by incubating the fraction in medium containing 60 mM KCl, 10 mM Tris - HCl, pH 7.0, 4 mM EGTA, and 3 mM $MgCl_2$, at a protein concentration of 0.8 to 1.0 mg/ml. The reaction at 37°C was initiated by the addition of 2 mM ATP. The total ATPase activity of the myofibrillar fraction was determined in the above medium without EGTA in the presence of 0.1 mM $CaCl_2$. The basal and $Ca^{++}$ - stimulated ATPase activities were calculated. The ATPase activity of myofibrils obtained by Procedure B was determined in the absence and presence of 10 mM sodium azide by incubating the

fraction in medium containing 50 mM KCl, 20 mM Tris - HCl, pH 7.0, 2 mM Mg $Cl_2$, 1.6 mM EGTA at a protein concentration of 1 to 2 mg/ml.

Sarcolemmal $Na^+ - K^+$ ATPase activity was determined in 1 ml of a medium containing 50 mM Tris - HCl, pH 7.4, 4 mM Mg $Cl_2$, 100 mM Na Cl and 10 mM KCl in the absence or presence of 2 mM ouabain. Sarcolemmal protein (0.02 to 0.05 mg/ml) was preincubated for 3 minutes at 37°C and the reaction was initiated by the addition of 4 mM ATP. The reaction was allowed to proceed a further 10 min. The difference of the activities in the absence and presence of ouabain is referred to as $Na^+ - K^+$ stimulated, $Mg^{++}$ dependent, ouabain sensitive ATPase.

The $Ca^{++}$ ATPase and $Mg^{++}$ ATPase activities were assayed in a total volume of 1 ml containing 50 mM Tris - HCl, pH 7.4, 4 mM Ca $Cl_2$ or Mg $Cl_2$ and 4 mM ATP. Membrane protein (0.02 to 0.05 mg/ml) was preincubated at 37°C for 3 min and the reaction was initiated by the addition of ATP. The reaction was allowed to proceed for a further 10 minutes. The ATP hydrolysis that occurred in the absence of $Ca^{++}$ or $Mg^{++}$ was substracted in order to calculate the activity due to $Ca^{++}$-stimulated or $Mg^{++}$ stimulated ATPase.

All fractions were preincubated for 3 min in the absence or presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ and the reactions for ATP hydrolysis were started by the addition of ATP and stopped by the addition of 12% cold trichloroacetic acid and centrifugation. The inorganic phosphate released in the clear supernatant was measured by the method of Taussky and Shorr (276). The protein concentration was determined by the method of Lowry et. al. (277).

(3) Determination of adenylate cyclase activity

The adenylate cyclase activity was assayed by the method of Drummond and Duncan (278). The sarcolemmal fraction (50 µg protein/0.15 ml) was pre-incubated in a medium containing 50 mM Tris - HCl, pH 8.5, 8 mM caffeine, 5 mM KCl, 20 mM phosphoenol pyruvate, 15 mM Mg $Cl_2$, 2 mM cyclic AMP and 130 µg/ml pyruvate kinase for 3 min at 37°C followed by the addition of 0.4 mM $^{14}C$ - ATP. The reaction was terminated by boiling and care was taken to avoid evaporation. Protein was removed by centrifugation at 1000 x g for 10 min and 50 µl of the clear

supernatant was spotted on Whatman No. 3 MM paper for descending chromatography, using 1 M ammonium acetate - 95% ethanol (15 : 35 v/v) as a solvent. The chromatograms were developed for 18 hours and after drying, the cyclic AMP area was visualized by ultraviolet light, cut out and counted in 20 ml of Bray's solution in a Packard Liquid Scintillation Spectrometer. Different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were added during the preincubation period.

(4) Determination of mitochondrial respiration and oxidative phosphorylation activities

The mitochondrial oxygen consumption and oxidative phosphorylation (ADP:O ratio) were measured polarographically at 28°C using a Gilson oxygraph and Clarke electrode in a medium containing 0.25 M sucrose, 10 mM Tris - HCl, pH 7.4, 10 mM $K_2HPO_4$, 1.5 mM pyruvate and 0.5 mM malate. State 3 respiration was initiated by the addition of 250 n moles ADP whereas state 4 respiration ensued when all the ADP was phosphorylated. The respiratory control index (RCI) was calculated as the ratio of oxygen uptake rates in states 3 and 4 whereas the phosphorylation rate was calculated by multiplying the oxygen uptake rate in state 3 ($QO_2$ (3)) by the ADP:O ratio. This method is essentially the same as that described by Sordahl and Schwartz (275). These activities were measured in the absence or presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$.

E. Analysis of Data

Each observation was made with 4 to 6 different preparations. The mean $\pm$ S.E. for each point 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 a significant difference between control and experimental results.

# IV. RESULTS

## A. Contractile Force Developement and Myocardial Ultrastructure

The effects of different concentrations (0.1 - 1 mM) of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were studied on the contractile force and the rate of change of contractile force developement (df/dt) of isolated perfused rabbit hearts. All these divalent cations were found to depress myocardial contractility to varying degrees depending upon their concentration in the perfusion medium. In 0.1, 0.5, and 1 mM concentrations the contractile force was decreased by about 25, 44 and 95% by $Co^{++}$, 40, 75 and 100% by $Ni^{++}$, and 28, 66 and 84% by $Mn^{++}$ respectively. The effects of 0.5 and 1 mM concentrations of these divalent cations on df/dt and contractile force are shown in Figures 1 - 3. It can be seen that the onset of cardiodepressent effects of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ was quite rapid. Furthermore, an increase in resting tension with 0.5 and 1 mM concentrations of $Co^{++}$ and $Ni^{++}$ was seen in all experiments whereas $Mn^{++}$ in 0.5 mM, but not in 1 mM, produced only a transient increase in resting tension.

When sections of the hearts perfused with 1 mM $Co^{++}$, $Ni^{++}$ or $Mn^{++}$ for 3 min were examined under the electron microscope, their ultrastructure (Figure 4) was found not to be different from the control.

## B. Myofibrillar ATPase Activities

Myofibrils from the rabbit hearts were isolated by two different procedures and their ATPase activities were examined in the absence or presence of sodium azide for determining the extent of mitochondrial contamination. The data given in Table 1 indicate that the $Mg^{++}$ ATPase activity of the preparation (Procedure A) obtained by the method of Muir et. al. (273), unlike the preparation (Procedure B) obtained by the method of Solaro et. al. (274), was sensitive to 5 mM sodium azide. Although $Ca^{++}$ - stimulated ATPase activity in both preparations was not affected by sodium azide, the activity of this enzyme in preparation B was significantly higher than that of preparation A. The actions of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on these preparations were similar; however, all the subsequent experiments described in this study were performed by employing preparation B and sodium azide, a well

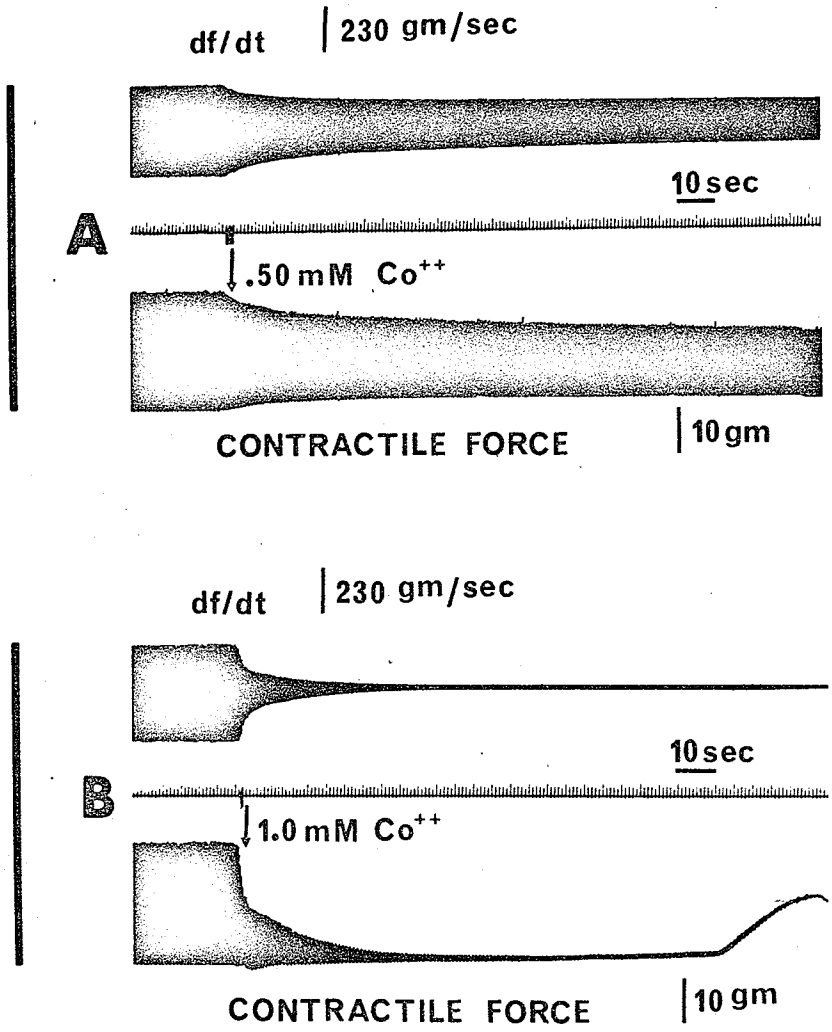



FIGURE 1

Representative tracings of force developement by the isolated perfused heart in the presence of different concentrations of $Co^{++}$. Panels A and B show the effects of 0.5 mM and 1.0 mM $Co^{++}$ respectively. The upper portion of each panel shows the rate of change of contractile force developement (df/dt) whereas the lower portion shows contractile force. These figures are typical of 3 experiments carried out for each concentration.

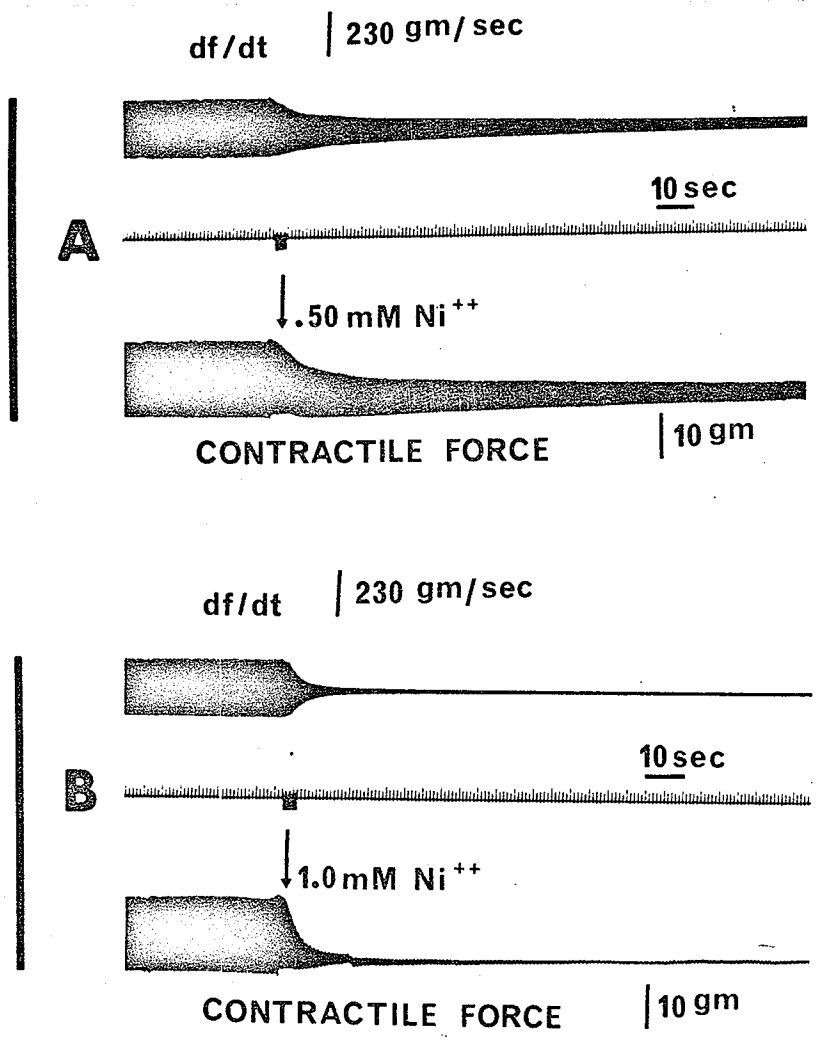



FIGURE 2

Representative tracings of force developement by the isolated perfused heart in the presence of different concentrations of $Ni^{++}$. Panels A and B show the effects of 0.5 mM and 1.0 mM $Ni^{++}$ respectively. The upper portion of each panel shows the rate of change of contractile force developement (df/dt) whereas the lower portion shows contractile force. These figures are typical of 3 experiments carried out for each concentration.

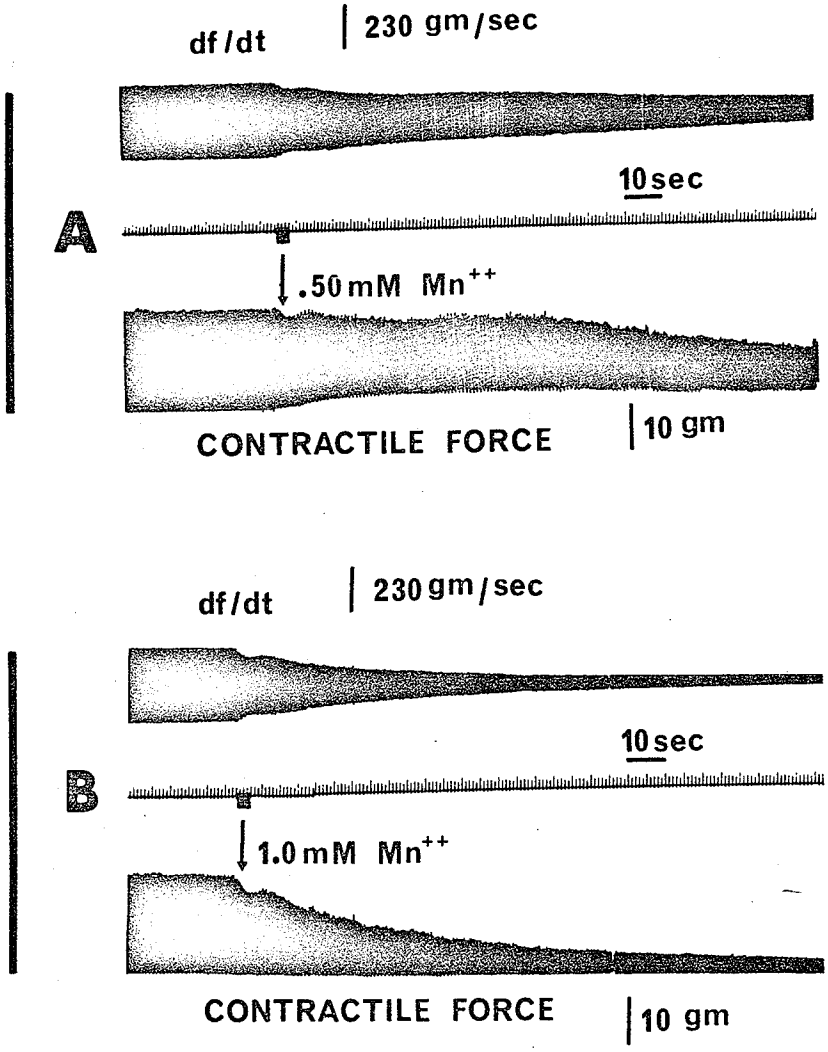



FIGURE 3

Representative tracings of force developement by the isolated perfused heart in the presence of different concentrations of $Mn^{++}$. Panels A and B show the effects of 0.5 mM and 1.0 mM $Mn^{++}$ respectively. The upper portion of each panel shows the rate of change of contractile force developement (df/dt) whereas the lower portion shows contractile force. These figures are typical of 3 experiments carried out for each concentration.

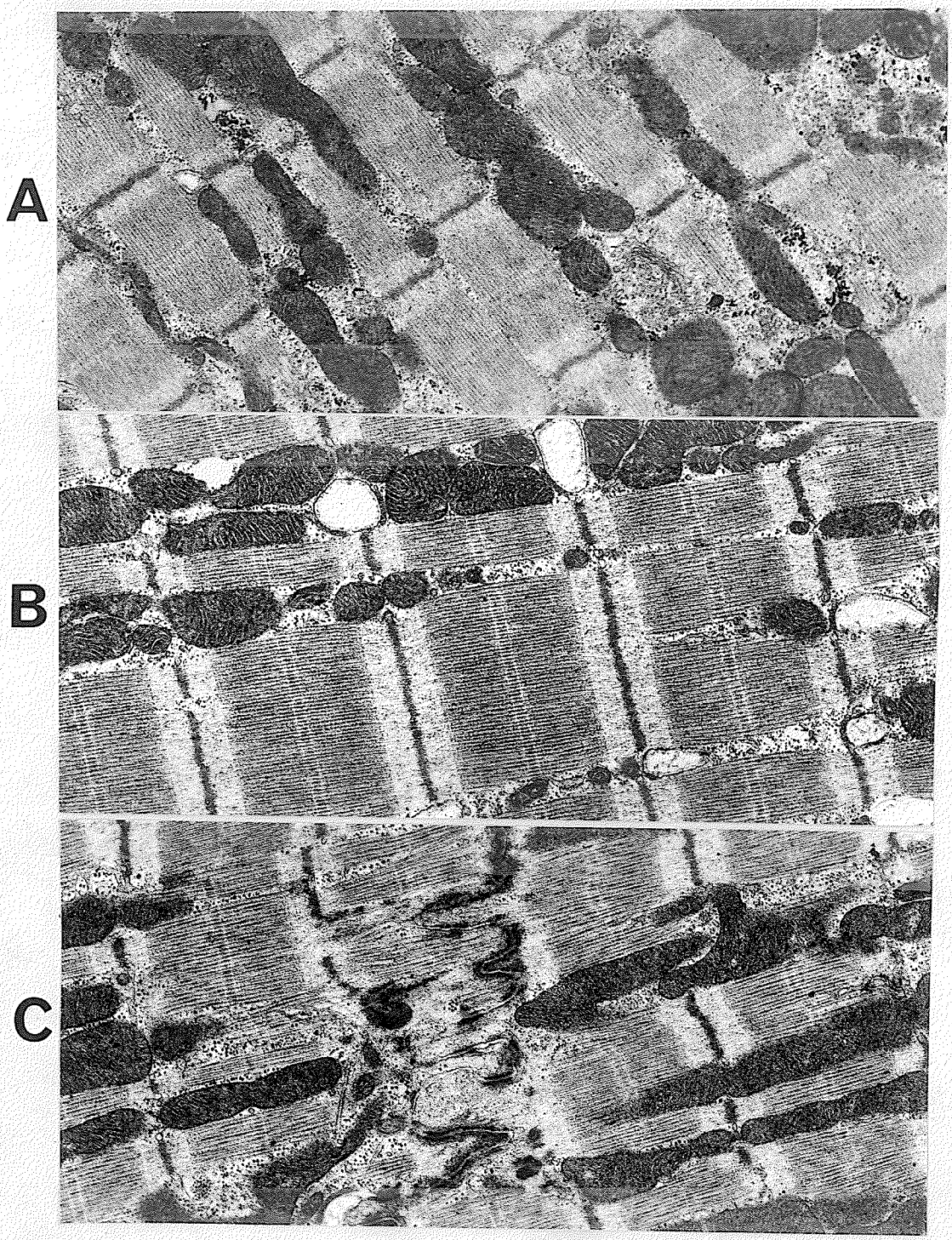



FIGURE 4 Electron photomicrograph of biopsies of left ventricle of $Co^{++}$ (panel A), $Ni^{++}$ (panel B), and $Mn^{++}$ (panel C) treated hearts. X26,500.

TABLE I

ATPase Activities of Myofibrils Isolated by Different Procedures

| Preparation | ATPase activity ($\mu$moles Pi/mg protein/min)* | | | |
|---|---|---|---|---|
| | $Mg^{++}$ - ATPase | | $Ca^{++}$ - stimulated ATPase | |
| | - Azide | +Azide | -Azide | +Azide |
| A | .124 ± .014 | .058 ± .006 | .127 ± .012 | .130 ± .011 |
| B | .033 ± .006 | .034 ± .004 | .185 ± .010 | .182 ± .008 |

* Procedures for preparation A and B are described in the Methods section and the ATPase activities were measured in the absence or presence of 5 mM sodium azide. The concentration of Mg ATP was 2 mM and that of $Ca^{++}$ was 0.1 mM. Each value is a mean ± S.E. of 4 experiments.

known inhibitor of mitochondrial ATPase, was used in the incubation medium.

In one series of experiments, the ability of myofibrils to hydrolyze ATP in the presence of $Ca^{++}$, $Mg^{++}$, $Co^{++}$, $Ni^{++}$ or $Mn^{++}$ was tested and the results are given in Table 2. Although $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were able to stimulate ATP hydrolysis by myofibrils, the extent of their action was less than that of either $Ca^{++}$ or $Mg^{++}$. The myofibrillar $Mg^{++}$ ATPase activity was also determined in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ and the results are shown in Figure 5. It can be seen that $Mg^{++}$ ATPase activity was inhibited by $Mn^{++}$, $Co^{++}$ and $Ni^{++}$ (0.05 - 1 mM) in this order of potency except that $Ni^{++}$ had no significant ($P > 0.05$) effect at a concentration of 0.05 mM. It should be pointed out here that in contrast to $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, 0.1 mM calcium produced a 5 - 6 fold increase in the myofibrillar $Mg^{++}$ ATPase activity.

The effects of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were also studied on the myofibrillar $Ca^{++}$-stimulated ATPase activity. The results in Figure 6 indicate that these divalent cations produced significant ($P < 0.05$) depressions in the $Ca^{++}$-stimulated ATPase activity in concentrations ranging from 0.05 to 1 mM. The order of potency for this inhibition was $Ni^{++} > Co^{++} > Mn^{++}$. When the effects of 1 mM $Co^{++}$, $Ni^{++}$ or $Mn^{++}$ on $Ca^{++}$ - stimulated ATPase were examined at different concentrations of Mg ATP, which serves as its substrate, it was found that in contrast to $Co^{++}$ or $Ni^{++}$, the inhibitory effect of $Mn^{++}$ was not apparent at 0.25 to 1 mM concentrations of Mg ATP (Table 3).

C. Mitochondrial ATPase, Calcium Accumulation and Oxidative Phosphorylation Activities

The ability of mitochondria to hydrolyze ATP in the presence of 0.05 - 4 mM concentrations of $Ca^{++}$, $Mg^{++}$, $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ was studied and the results are shown in Table 4. All these divalent cations were found to stimulate ATP hydrolysis and their order of potency was $Mn^{++} = Mg^{++} > Co^{++} > Ca^{++} > Ni^{++}$. In another set of experiments the effect of different concentrations (0.25 to 4 mM) of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were examined on mitochondrial ATPase activity in the presence of $Mg^{++}$. From the results in Figure 7, it can be seen that the mitochondrial $Mg^{++}$ ATPase activity was slightly depressed ($P < 0.05$) by 1 - 4 mM concentrations

TABLE II

Myofibrillar ATP Hydrolysis in the Presence of Different Concentrations of Divalent Cations

| Concentrations of cation (mM) | ATP hydrolysis (μmoles Pi/mg protein/min)* | | | | |
|---|---|---|---|---|---|
| | $Ca^{++}$ | $Mg^{++}$ | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| .01 | .122 ± .008 | .120 ± .008 | .088 ± .006 | .076 ± .004 | .080 ± .004 |
| .05 | .129 ± .009 | .120 ± .008 | .082 ± .006 | .082 ± .006 | .080 ± .004 |
| .10 | .134 ± .008 | .115 ± .007 | .083 ± .005 | .080 ± .003 | .090 ± .006 |
| .25 | .159 ± .006 | .096 ± .007 | .084 ± .004 | .079 ± .006 | .094 ± .009 |
| .50 | .170 ± .010 | .087 ± .006 | .088 ± .009 | .079 ± .004 | .096 ± .009 |

* The incubation medium was the same as that described for myofibrillar ATPase activity in the Methods section except that EGTA was omitted. The value for myofibrillar ATP hydrolysis in the absence of any added cation was .037 ± .004 μmoles Pi/mg protein/min. The concentration of ATP was 2 mM. Each value is a mean ± S.E. of 3 experiments.

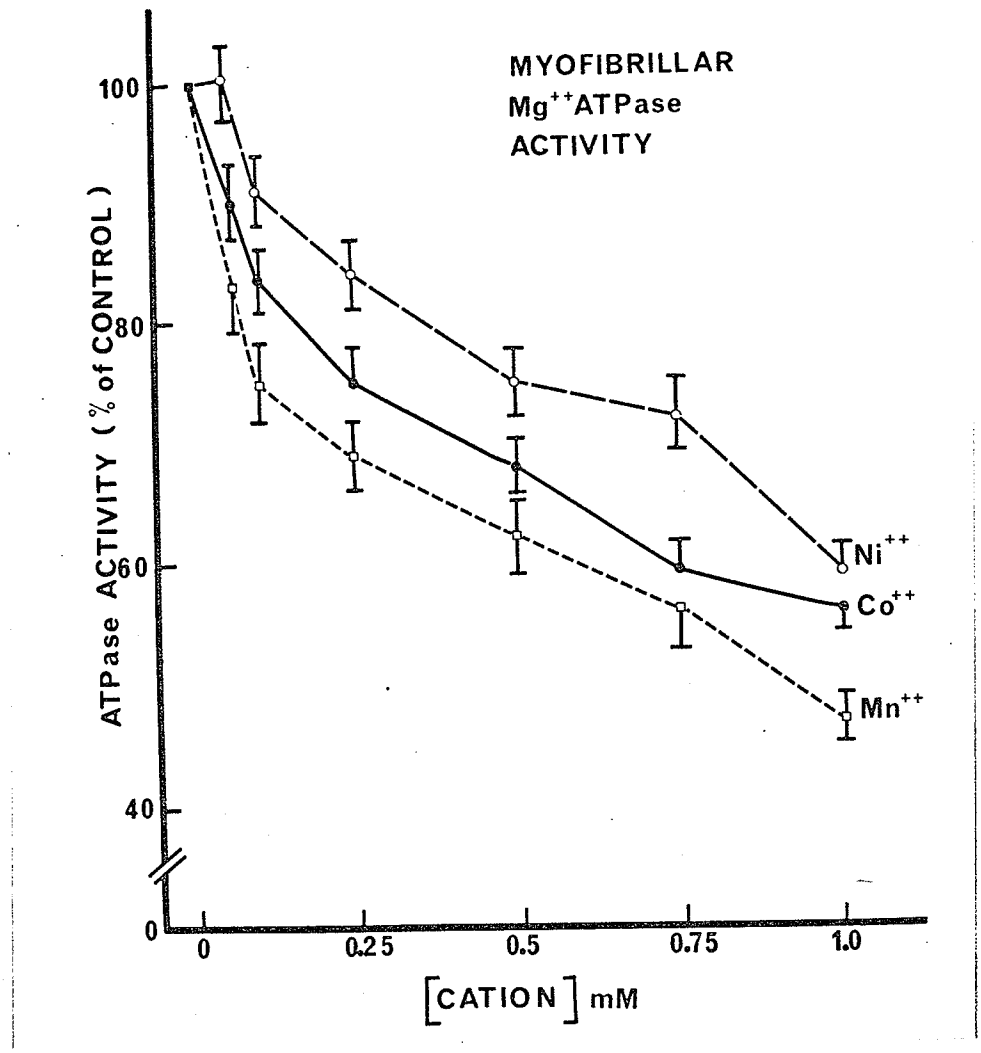



FIGURE 5

Myofibrillar $Mg^{++}$ ATPase activity in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The incubation medium was the same as that described in the Methods section. The control value for myofibrillar ATPase activity was .032 + .003 μ moles Pi/mg protein/min. Each value is a mean $\pm$ S.E. of 4 experiments.

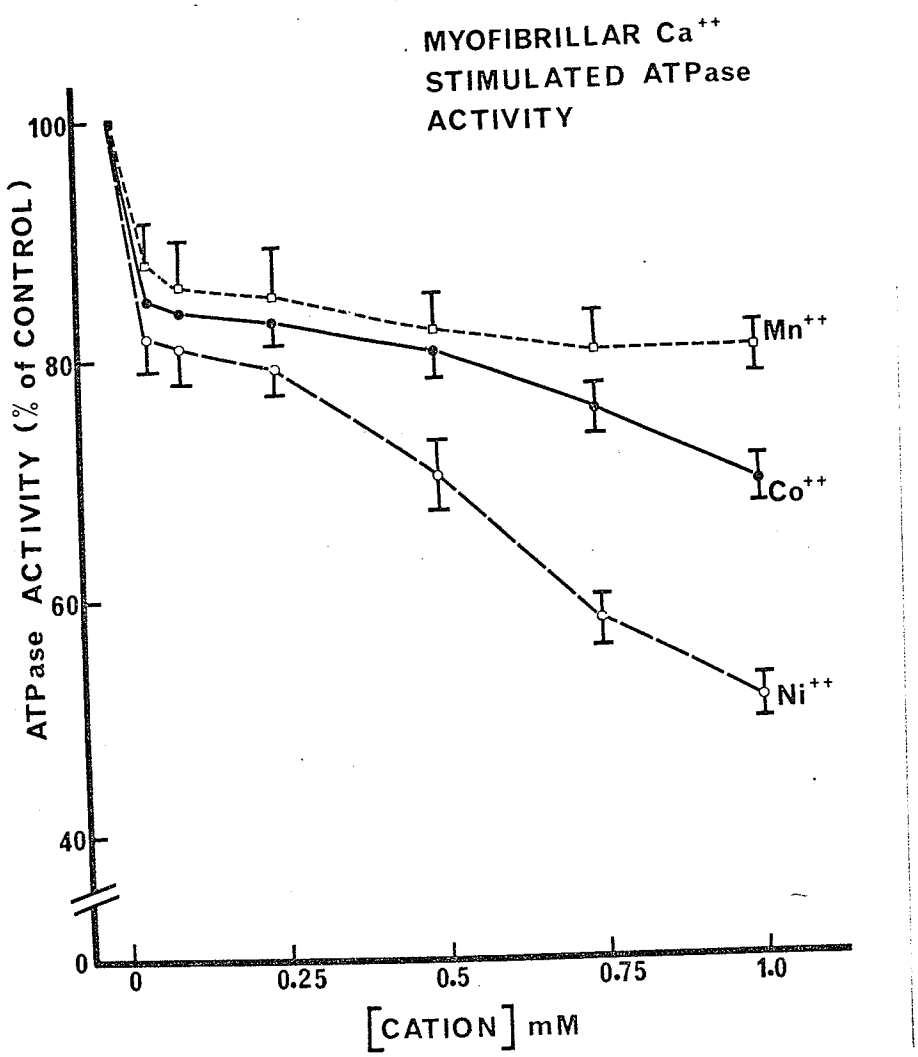



FIGURE 6

Myofibrillar $Ca^{++}$ - stimulated ATPase activity in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The incubation medium was the same as that described in the Methods section. The control value for myofibrillar $Ca^{++}$ - stimulated ATPase activity was .186 + .014 μ moles Pi/mg protein/min. Each value is a mean $\pm$ S.E. of 4 experiments.

TABLE III

Effect of 1 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on Myofibrillar $Ca^{++}$ - stimulated ATPase Activity in the Presence of Different Concentrations of Mg ATP

| Concentration of Mg ATP (mM) | $Ca^{++}$ - stimulated ATPase (μmoles Pi/mg protein/min)** | | | |
|---|---|---|---|---|
| | Control | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| .25 | .034 ± .004 | .022 ± .002 | .007 ± .003 | .034 ± .002* |
| .50 | .064 ± .002 | .048 ± .006 | .015 ± .001 | .063 ± .001* |
| .75 | .082 ± .006 | .065 ± .005 | .029 ± .002 | .078 ± .006* |
| 1.0 | .109 ± .005 | .079 ± .005 | .048 ± .004 | .105 ± .008* |
| 2.0 | .178 ± .014 | .108 ± .008 | .078 ± .005 | .150 ± .005 |

* Not significantly different from control ($P > 0.05$)

** The incubation medium was the same as that described in the Methods section except that different concentrations of Mg ATP were employed. Each value is a mean ± S.E. of 4 experiments.

TABLE IV

Mitochondrial ATP Hydrolysis in the Presence of Different Concentrations of Divalent Cations

| Concentrations of cation (mM) | ATP hydrolysis (μmoles Pi/mg protein/min)* | | | | |
|---|---|---|---|---|---|
| | $Ca^{++}$ | $Mg^{++}$ | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| .05 | 0.12 ± .01 | 0.12 ± .01 | 0.14 ± .02 | 0.14 ± .02 | 0.15 ± .03 |
| .10 | 0.21 ± .01 | 0.15 ± .02 | 0.18 ± .04 | 0.16 ± .02 | 0.20 ± .02 |
| .25 | 0.26 ± .01 | 0.23 ± .02 | 0.23 ± .05 | 0.18 ± .03 | 0.26 ± .01 |
| .50 | 0.28 ± .02 | 0.32 ± .02 | 0.37 ± .03 | 0.21 ± .01 | 0.40 ± .04 |
| 1.0 | 0.33 ± .03 | 0.68 ± .05 | 0.60 ± .04 | 0.28 ± .03 | 0.80 ± .06 |
| 2.0 | 0.35 ± .03 | 1.00 ± .07 | 0.90 ± .07 | 0.30 ± .03 | 1.10 ± .07 |
| 4.0 | 0.38 ± .03 | 1.05 ± .06 | 0.78 ± .06 | 0.29 ± .04 | 1.01 ± .05 |

*The incubation medium was the same as that described for mitochondrial ATPase activity in the Methods section. The value for mitochondrial ATP hydrolysis in the absence of any added cation was 0.12 ± .02 μmoles Pi/mg protein/min. The concentration of ATP was 4 mM. Each value is a mean ± S.E. of 4 experiments.

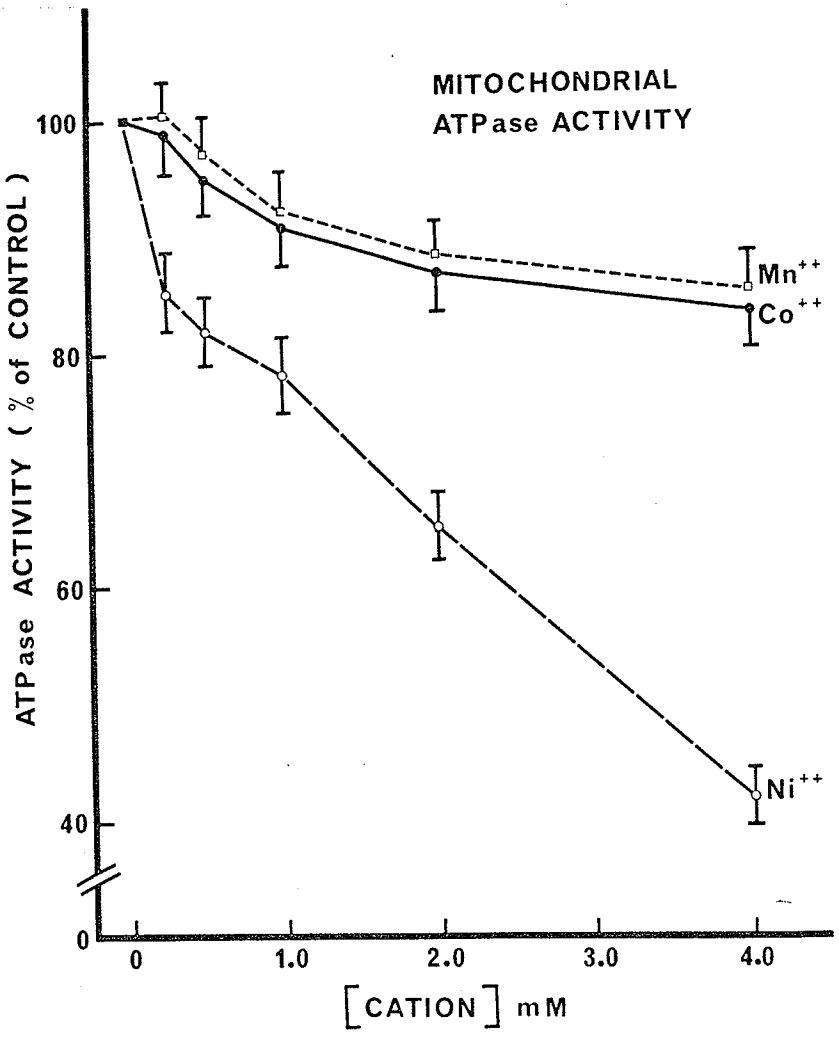



FIGURE 7

Mitochondrial ATPase activity in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The incubation medium was the same as that described in the Methods section. The control value for mitochondrial ATPase activity was 1.0 ± .06 μ moles Pi/mg protein/min. Each value is a mean ± S.E. of 4 experiments.

of $Mn^{++}$ and $Co^{++}$ whereas a marked reduction was observed by 0.25 to 4 mM concentrations of $Ni^{++}$. It should be mentioned that mitochondrial $Mg^{++}$ ATPase activity was not increased by low concentrations (0.05 - 0.1 mM) of calcium indicating the absence of $Ca^{++}$ - stimulated ATPase activity in this fraction.

The energy - linked ability of mitochondria to accumulate calcium in the absence (calcium binding) and presence (calcium uptake) of phosphate was examined under different experimental conditions. The calcium binding activity of mitochondria in the presence of ATP was found to be $52 \pm 3.8$ n moles/mg protein/5 min; 80 - 90% of this activity was inhibited by 5 mM sodium azide. The ATP - dependent calcium uptake activities of mitochondria in the absence and presence of succinate were $131 \pm 7.4$ and $176 \pm 6.5$ n moles/mg protein/5 min respectively. The substrate - linked (pyruvate - malate) calcium uptake activity of mitochondria was found to be $186 \pm 12.9$ n moles/mg protein/5 min. It should be pointed out that calcium binding and uptake by mitochondria represent arbitary meanings of these terms and are employed here for the purpose of clarity.

Figure 8 shows the effect of different concentrations of divalent cations on mitochondrial calcium binding. Both $Co^{++}$ and $Ni^{++}$ were found to decrease ($P < 0.05$) calcium binding activity of mitochondria at 0.01 to 1 mM concentrations whereas a significant ($P < 0.05$) depression by $Mn^{++}$ was apparent at 0.1 mM concentration. Similar depressant effects of these divalent cations were seen on the ATP - dependent, ATP - substrate supported, and substrate - supported calcium uptake activities of mitochondria (Figures 9 - 11) with some minor differences with respect to the effective concentrations of cations and the magnitude of depression. In this regard it should be noted that calcium uptake under different experimental conditions was decreased by $Mn^{++}$ at 0.25 mM concentration whereas depressant effect of $Co^{++}$ on the substrate - supported calcium uptake was apparent at 0.1 mM concentration. The order of their potency in decreasing mitochondrial calcium binding and uptake activities was $Ni^{++} > Co^{++} > Mn^{++}$. Furthermore, the magnitudes of depressant effects of both $Ni^{++}$ and $Co^{++}$ on calcium binding and ATP - substrate supported calcium uptake were greater than those on the ATP or substrate - supported calcium uptake. On the other hand, the depressant effect of $Mn^{++}$ on calcium binding

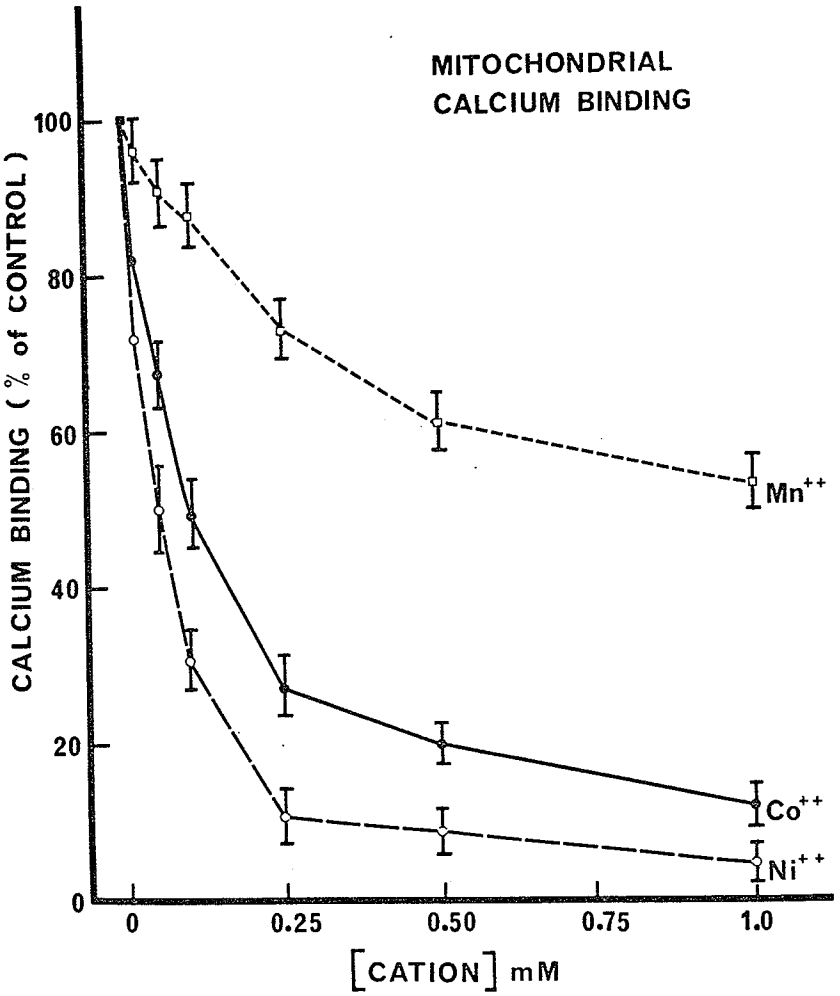



FIGURE 8

Mitochondrial calcium binding in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The time of incubation of the mitochondrial fraction with 0.1 mM $^{45}Ca^{++}$ at 25° C was 5 min. The control value for calcium binding was 52 ± 3.8 n moles/mg protein. Each value is a mean ± S.E. of 4 experiments.

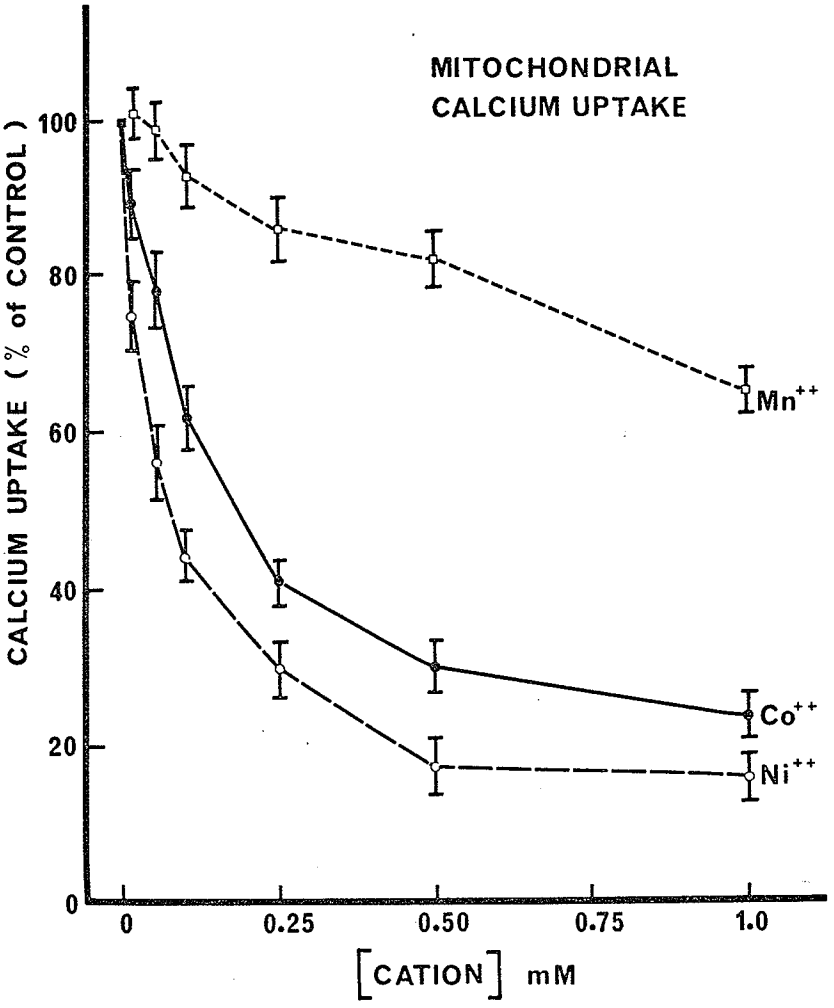



FIGURE 9

ATP - supported mitochondrial calcium uptake in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The time of incubation of the mitochondrial fraction with 0.1 mM $^{45}Ca^{++}$ at 37°C was 5 min. The control value for calcium uptake was 131 + 7.4 n moles/mg protein. Each value is a mean $\pm$ S.E. of $\overline{4}$ experiments.

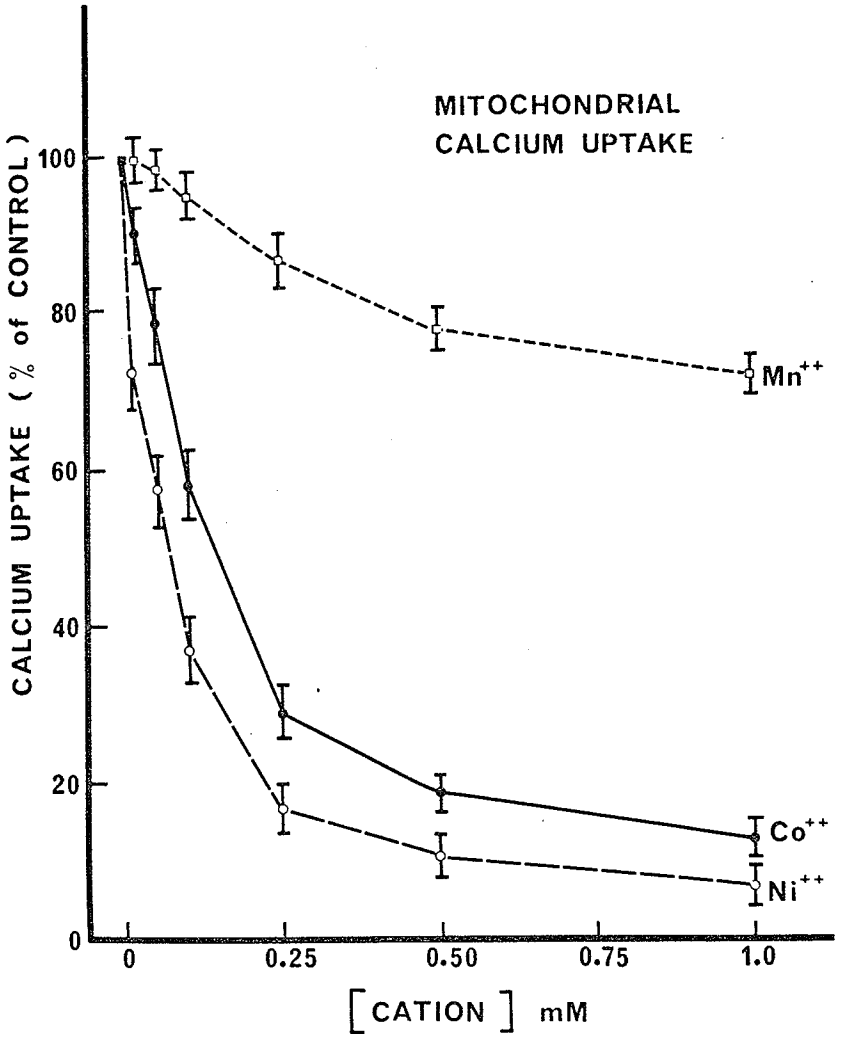



FIGURE 10 ATP - succinate - supported mitochondrial calcium uptake in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The time of incubation of the mitochondrial fraction with 0.1 mM $^{45}Ca^{++}$ at 37°C was 5 min. The control value for calcium uptake was 176 + 6.5 n moles/mg protein. Each value is a mean $\pm$ S.E. of 4 experiments.

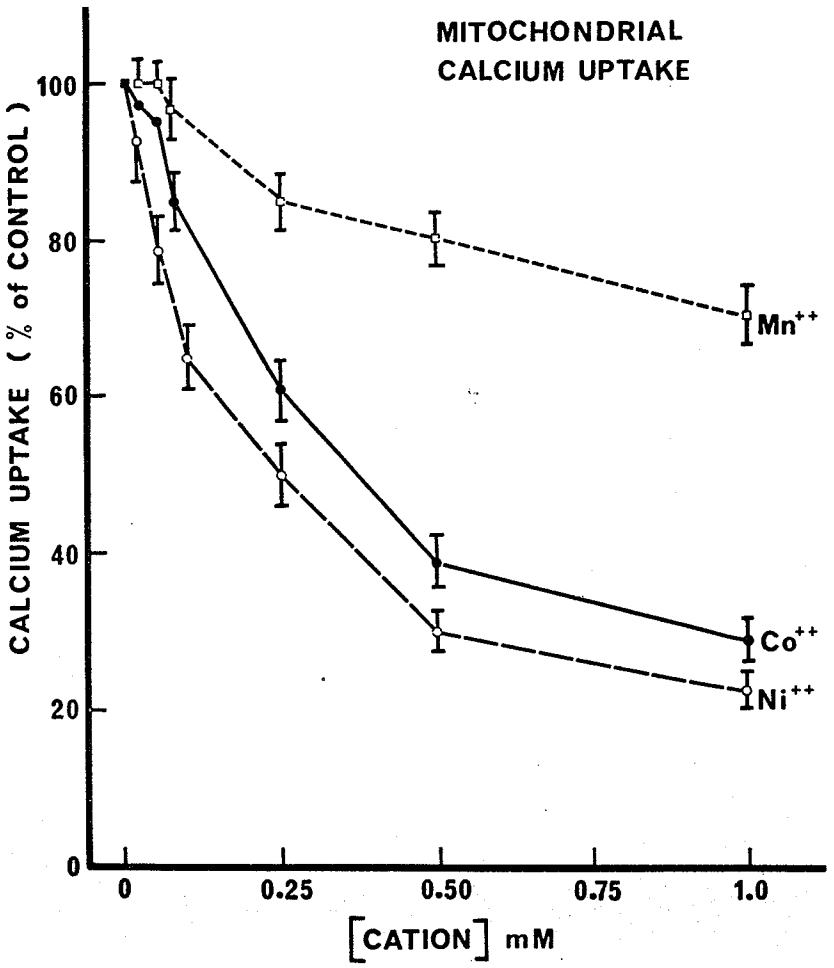



FIGURE 11

Pyruvate - malate - supported mitochondrial calcium uptake in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The time of incubation of the mitochondrial fraction with 0.1 mM $^{45}Ca^{++}$ at 37°C was 5 min. The control value for calcium uptake was 186 ± 12.9 n moles/mg protein. Each value is a mean ± S.E. of 4 experiments.

was greater than that on calcium uptake activities under different experimental conditions.

A time - course concerning the effects of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on the ATP - substrate supported mitochondrial calcium uptake revealed that the depressent effects of these divalent cations were apparent at different intervals (30 sec - 10 min) of incubation (Table 5). When the effects of these divalent cations (0.1 mM) were studied by employing different concentrations of calcium (10 - 75 μM), it was found that both $Co^{++}$ and $Ni^{++}$, unlike $Mn^{++}$, produced depressant effects at all concentrations of calcium employed in the incubation medium (Table 6). $Mn^{++}$ at 0.25 mM concentrations was found to have inhibitory effects on calcium uptake at all concentrations of $Ca^{++}$ in the incubation medium.

The effects of different concentrations of divalent cations on the mitochondrial respiration and oxidative phosphorylation activities were also studied. It can be seen from Tables 7 - 9 that the effect of these cations were apparent at 50 - 100 μM concentrations. $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ increased state 4 respiration and decreased RCI and ADP:O ratio. The state 3 respiration was decreased by $Co^{++}$ but increased by $Ni^{++}$ and $Mn^{++}$ whereas the phosphorylation rate was decreased by $Co^{++}$ and was not affected by $Ni^{++}$ or $Mn^{++}$. It was interesting to observe that concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ greater than 0.1 mM uncoupled the mitochondrial respiratory activities completely.

D. Microsomal ATPase and Calcium Accumulation Activities

Microsomal fraction is known to possess basal ($Mg^{++}$ ATPase) and $Ca^{++}$-stimulated ATPase activities. In the first series of experiments, the abilities of $Ca^{++}$, $Mg^{++}$, $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ to stimulate ATP hydrolysis by this fraction were compared. The results shown in Table 10 indicate that all these divalent cations were capable of stimulating ATP hydrolysis in 0.1 - 4 mM concentrations except $Ni^{++}$ which was effective at 1 - 4 mM concentrations. The order of their potency was $Mg^{++} > Mn^{++} > Ca^{++} > Co^{++} > Ni^{++}$. The $Mg^{++}$ ATPase activity (1.41 μ moles Pi/mg protein/min) of the microsomal fraction was inhibited (Figure 12) significantly ($P < 0.05$) by 0.25 - 4 mM $Ni^{++}$ or $Co^{++}$ and by 0.5 - 4 mM $Mn^{++}$. The order of potency for inhibiting $Mg^{++}$ ATPase activity was $Ni^{++} > Co^{++} >$

TABLE V

Time Course of Mitochondrial Calcium Uptake in the Absence and Presence of 0.25 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$

| Incubation time | Mitochondrial calcium (nmoles/mg protein)* | | | |
|---|---|---|---|---|
| | Control | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| 30 sec | 28 ± 1.5 | 18 ± 1.1 | 15 ± 1.6 | 21 ± 1.8 |
| 1 min | 51 ± 2.5 | 21 ± 1.3 | 16 ± 0.9 | 38 ± 2.3 |
| 2 min | 85 ± 2.4 | 27 ± 1.5 | 19 ± 1.0 | 74 ± 3.3 |
| 5 min | 168 ± 4.8 | 49 ± 2.5 | 32 ± 1.2 | 133 ± 4.8 |
| 10 min | 254 ± 7.3 | 75 ± 4.2 | 44 ± 1.8 | 208 ± 3.7 |

* The incubation medium was the same as that described for mitochondrial calcium uptake in the presence of 4 mM ATP and 5 mM sodium succinate in the Methods section. Mitochondria were incubated in the presence of 0.1 mM $^{45}Ca^{++}$ for various time intervals. Each value is a mean ± S.E. of 4 experiments. All values in the presence of divalent cations were significantly different ($P < 0.05$) from the control.

TABLE VI

Effect of 0.1 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on Mitochondrial Calcium Uptake in the Presence of Different Concentrations of Calcium

| | Mitochondrial calcium (nmoles/mg protein)** | | | |
|---|---|---|---|---|
| Calcium (μM) | Control | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| 10 | 8 ± 0.6 | 5 ± 0.5* | 5 ± 0.7* | 8 ± 0.7 |
| 25 | 27 ± 1.4 | 15 ± 1.1* | 9 ± 0.6* | 28 ± 1.3 |
| 50 | 66 ± 2.1 | 45 ± 2.2* | 27 ± 1.2* | 66 ± 3.2 |
| 75 | 103 ± 4.6 | 78 ± 3.7* | 58 ± 2.7* | 106 ± 5.8 |

* Significantly different from control ($P < 0.05$).

** The incubation medium was the same as that described for mitochondrial calcium uptake in the presence of 4 mM ATP and 5 mM sodium succinate in the Methods section except that various concentrations of $Ca^{++}$ were employed. Each value is a mean ± S.E. of 4 experiments.

TABLE VII

Effect of Different Concentrations of $Co^{++}$ on Mitochondrial Respiration and Oxidative Phosphorylation Activities

| Concentration of $Co^{++}$ (μ M) | $O_2$ consumption (n atoms O/mg protein/min) | | RCI | ADP:O | Phosphorylation rate** (n moles ADP/mg protein/min) |
|---|---|---|---|---|---|
| | State 3 | State 4 | | | |
| 0 | 157 ± 5.4 | 14.2 ± 1.6 | 11.5 ± 1.4 | 2.8 ± 0.02 | 441 ± 14.4 |
| 10 | 159 ± 3.8 | 16.2 ± 1.6 | 10.4 ± 1.2 | 2.7 ± 0.04 | 429 ± 15.0 |
| 25 | 155 ± 4.6 | 17.2 ± 2.0 | 9.4 ± 1.1 | 2.7 ± 0.05 | 414 ± 17.2 |
| 50 | 149 ± 3.0* | 19.8 ± 3.2* | 8.2 ± 0.9* | 2.6 ± 0.06* | 387 ± 6.8* |
| 100 | 147 ± 2.6* | 28.8 ± 3.6* | 5.1 ± 0.4* | 2.5 ± 0.07* | 367 ± 6.2* |

* Significantly different from control ($P < 0.05$).

** State 3 respiration was initiated by the addition of a limited amount of ADP (250 n moles) whereas State 4 respiration ensued when all the ADP was phosphorylated. Each value is a mean ± S.E. of 4 experiments.

TABLE VIII

Effect of Different Concentrations of $Ni^{++}$ on Mitochondrial Respiration and Oxidative Phosphorylation Activities

| Concentration of $Ni^{++}$ ($\mu$ M) | $O_2$ consumption (n atoms O/mg protein/min) | | RCI | ADP:O | Phosphorylation rate** (n moles ADP/mg protein/min) |
|---|---|---|---|---|---|
| | State 3 | State 4 | | | |
| 0 | 158 ± 5.0 | 14.6 ± 1.2 | 10.9 ± 1.1 | 2.8 ± 0.02 | 444 ± 13.6 |
| 10 | 162 ± 3.4 | 18.4 ± 2.2 | 9.4 ± 1.1 | 2.7 ± 0.02 | 441 ± 10.4 |
| 25 | 165 ± 3.8 | 21.6 ± 3.0 | 8.6 ± 1.0 | 2.6 ± 0.04 | 436 ± 12.1 |
| 50 | 167 ± 4.4 | 24.0 ± 3.2* | 7.8 ± 0.8* | 2.5 ± 0.06* | 430 ± 9.7 |
| 100 | 176 ± 3.6 | 34.8 ± 3.4* | 5.6 ± 1.1* | 2.5 ± 0.08* | 433 ± 10.1 |

* Significantly different from control ($P < 0.05$).

** State 3 respiration was initiated by the addition of a limited amount of ADP (250 n moles) whereas State 4 respiration ensued when all the ADP was phosphorylated. Each value is a mean ± S.E. of 4 experiments.

TABLE IX

Effect of Different Concentrations of $Mn^{++}$ on Mitochondrial Respiration and Oxidative Phosphorylation Activities

| Concentration of $Mn^{++}$ ($\mu$ M) | $O_2$ consumption (n atoms O/mg protein/min) | | RCI | ADP:O | Phosphorylation rate** (n moles ADP/mg protein/min) |
|---|---|---|---|---|---|
| | State 3 | State 4 | | | |
| 0 | 156 ± 4.4 | 14.8 ± 1.4 | 10.5 ± 1.0 | 2.8 ± 0.02 | 436 ± 16.0 |
| 10 | 157 ± 4.2 | 18.0 ± 1.8 | 9.1 ± 1.0 | 2.7 ± 0.06 | 420 ± 20.0 |
| 25 | 165 ± 5.2 | 18.6 ± 2.2 | 8.8 ± 1.1 | 2.7 ± 0.09 | 432 ± 22.6 |
| 50 | 166 ± 5.0 | 26.4 ± 2.4* | 6.7 ± 1.0* | 2.5 ± 0.06* | 413 ± 15.4 |
| 100 | 173 ± 4.2* | 32.0 ± 2.4* | 5.8 ± 0.8* | 2.4 ± 0.04* | 421 ± 17.0 |

* Significantly different from control ($P < 0.05$).

** State 3 respiration was initiated by the addition of a limited amount of ADP (250 n moles) whereas State 4 respiration ensued when all the ADP was phosphorylated. Each value is a mean ± S.E. of 4 experiments.

TABLE X

Heavy Microsomal ATP Hydrolysis in the Presence of Different Concentrations of Divalent Cations

| Concentration of cation (mM) | ATP hydrolysis (μmoles Pi/mg protein/min)* | | | | |
|---|---|---|---|---|---|
| | $Ca^{++}$ | $Mg^{++}$ | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| .10 | .25 ± .02 | .30 ± .02 | .26 ± .03 | .18 ± .02 | .26 ± .01 |
| .25 | .34 ± .02 | .38 ± .04 | .34 ± .04 | .17 ± .03 | .47 ± .04 |
| .50 | .44 ± .03 | .57 ± .05 | .44 ± .03 | .20 ± .04 | .58 ± .06 |
| 1.0 | .54 ± .04 | .76 ± .04 | .57 ± .03 | .27 ± .03 | .79 ± .06 |
| 2.0 | .68 ± .04 | .92 ± .07 | .53 ± .01 | .26 ± .02 | .86 ± .04 |
| 4.0 | .74 ± .05 | .91 ± .04 | .40 ± .02 | .28 ± .03 | .72 ± .06 |

* The incubation medium was the same as that described for heavy microsomal ATPase activity in the Methods section except EGTA was omitted. The value for heavy microsomal ATP hydrolysis in the absence of any added cation was 0.18 ± .03 μmoles Pi/mg protein/min. The concentration of ATP was 4 mM. Each value is a mean ± S.E. of 4 experiments.

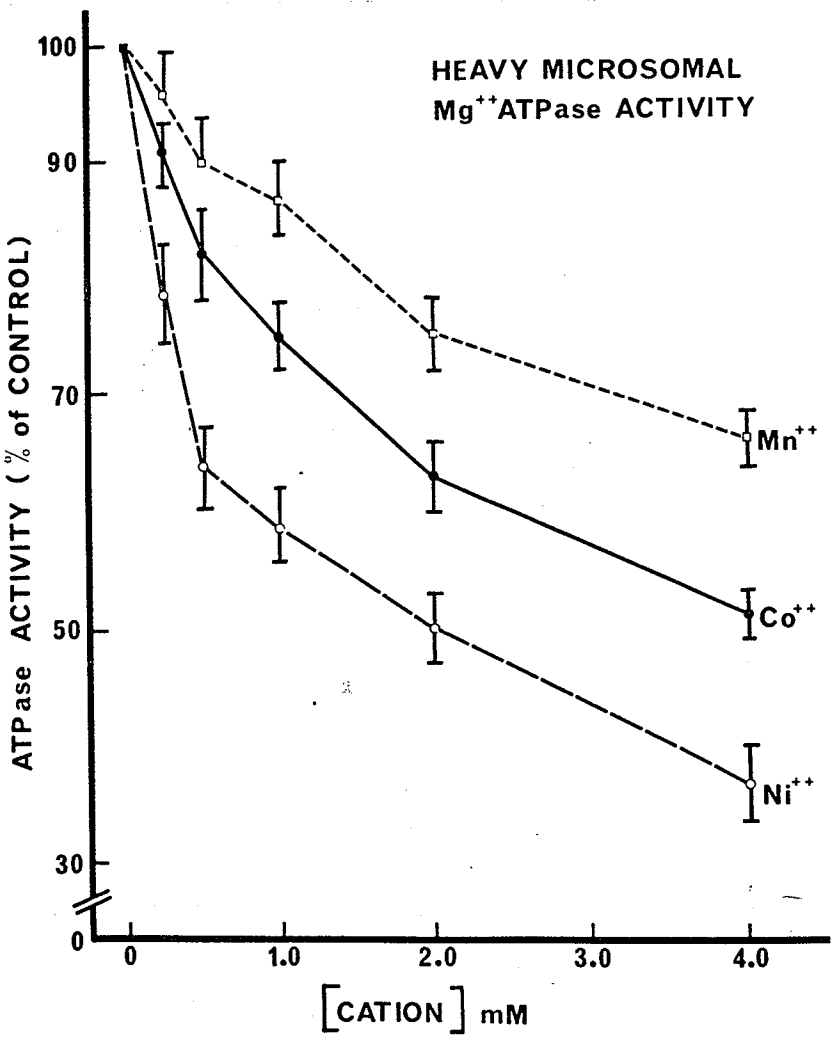



FIGURE 12

Heavy microsomal $Mg^{++}$ ATPase (basal) activity in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The incubation medium was the same as that described in the Methods section. The reaction was initiated by the addition of 4 mM ATP and carried out for 5 min. The control value for heavy microsomal $Mg^{++}$ ATPase activity was 1.41 + .05 μ moles Pi/ mg protein/min. Each value is a mean $\pm$ S.E. of 4 - 6 experiments.

$Mn^{++}$.

The $Mg^{++}$ ATPase activity of the microsomal fraction was stimulated by 0.1 mM $Ca^{++}$ and this activity due to $Ca^{++}$ - stimulated ATPase was found to be 0.44 μ moles Pi/mg protein/min. The activity of $Ca^{++}$ - stimulated ATPase was significantly decreased ($P < 0.05$) by 0.25 mM concentrations of $Ni^{++}$, $Co^{++}$ and $Mn^{++}$ and the order of their potency was $Ni^{++} > Co^{++} > Mn^{++}$ (Figure 13).

Heart microsomes accumulate calcium by an energy - dependent mechanism in the absence (calcium binding) and presence (calcium uptake) of permeant anions such as oxalate. The values for calcium binding and uptake activities for the rabbit heart microsomal fraction were found to be 45 and 1064 n moles/mg protein/5 min respectively. When calcium binding by microsomes was determined in the presence of different concentrations (0.1 - 2 mM) of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, these divalent cations were found to have no significant ($P > 0.05$) effect (Table 11). On the other hand, these cations had marked depressant action on the microsomal calcium uptake activity. Significant ($P < 0.05$) depression by $Ni^{++}$ was seen at 0.1 mM concentration whereas that by $Mn^{++}$ and $Co^{++}$ was observed at 0.25 and 0.50 mM respectively. The order of their potency was $Ni^{++} > Mn^{++} > Co^{++}$ (Figure 14).

The time - course of the inhibitory effects of 1 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ was also examined by incubating the microsomes for different intervals. It can be seen from Table 12 that these divalent cations produced significant ($P < 0.05$) depression in calcium uptake at 1 to 10 min but were ineffective at 30 sec of incubation. These divalent cations also decreased microsomal calcium uptake activities significantly ($P < 0.05$) when determined by employing different concentrations of calcium (10 - 75 μM) in the incubation medium (Table 13).

E. Sarcolemmal ATPase, Calcium Binding and Adenylate Cyclase Activities

The effects of different concentrations of $Ca^{++}$, $Mg^{++}$, $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on the ability of the sarcolemmal fraction to hydrolyze ATP were compared and the results are shown in Table 14. All these cations were capable of stimulating ATP hydrolysis by heart sarcolemma; $Ni^{++}$ was found to be least effective. The maximal $Ca^{++}$ ATPase and $Mg^{++}$ ATPase activities were found to be 22.8 and 21.6

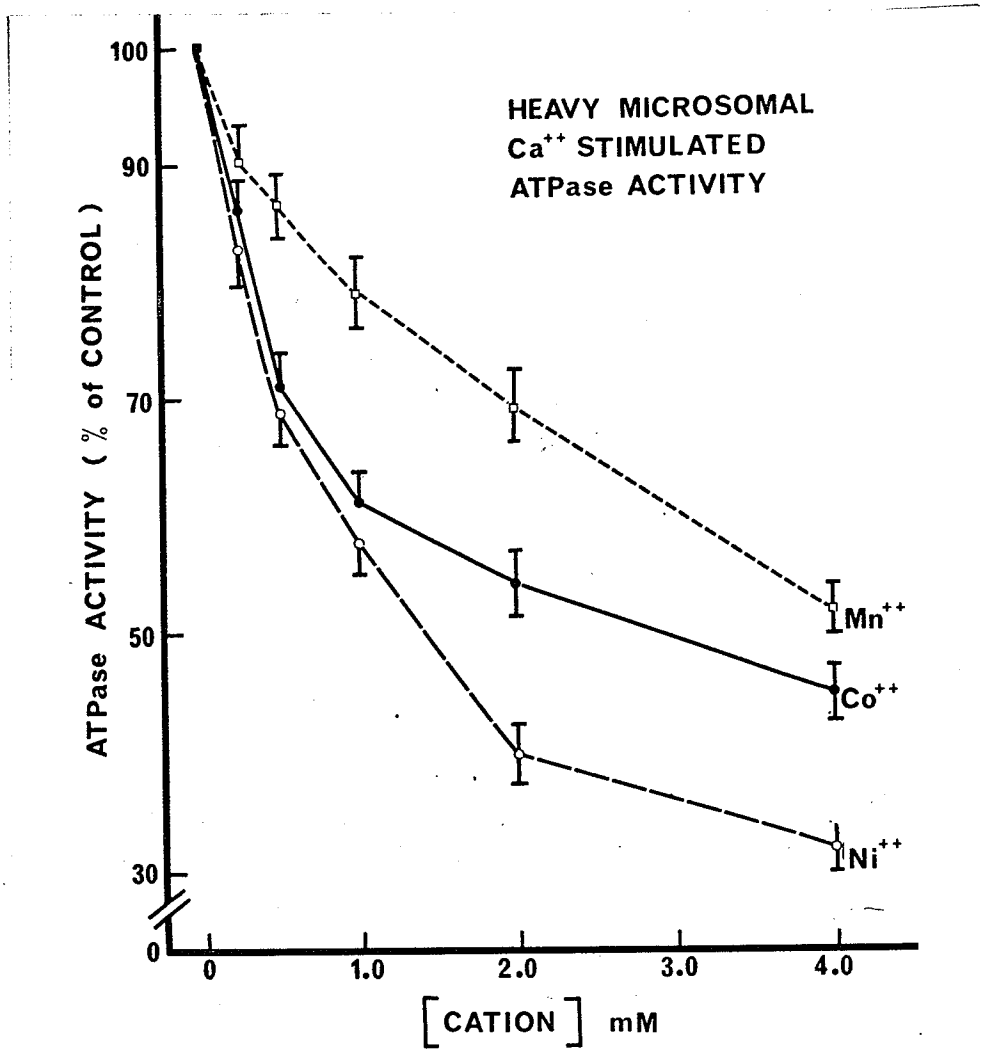



FIGURE 13

Heavy microsomal $Ca^{++}$ - stimulated ATPase activity in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The incubation medium was the same as that described in the Methods section. The control value for heavy microsomal $Ca^{++}$ - stimulated ATPase activity was 0.44 + .03 μ moles Pi/mg protein/min. Each value is a mean $\pm$ S.E. of 4 - 6 experiments.

TABLE XI

Heavy Microsomal Calcium Binding in the Absence and Presence of Different Concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$

| Concentration of cation (mM) | Heavy microsomal calcium* (nmoles/mg protein) | | |
|---|---|---|---|
| | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| 0 | 43.6 ± 1.7 | 46.3 ± 2.4 | 44.5 ± 2.7 |
| .10 | 46.0 ± 2.6 | 44.6 ± 3.6 | 42.7 ± 3.5 |
| .25 | 45.4 ± 2.3 | 45.0 ± 3.1 | 43.4 ± 2.4 |
| .50 | 42.9 ± 2.9 | 44.3 ± 2.9 | 44.9 ± 2.9 |
| .75 | 43.2 ± 3.3 | 42.7 ± 3.2 | 42.3 ± 2.5 |
| 1.0 | 40.7 ± 3.1 | 40.8 ± 2.5 | 44.1 ± 2.8 |
| 2.0 | 39.5 ± 2.1 | 41.6 ± 2.1 | 41.6 ± 2.4 |

* The incubation medium was the same as that described for calcium binding in the Methods section. Heavy microsomes were incubated in the presence of 0.1 mM $^{45}Ca^{++}$ for 5 minutes at 25°C. Each value is a mean ± S.E. of 3 experiments. None of the values for calcium binding in the presence of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were significantly different from control ($P > 0.05$).

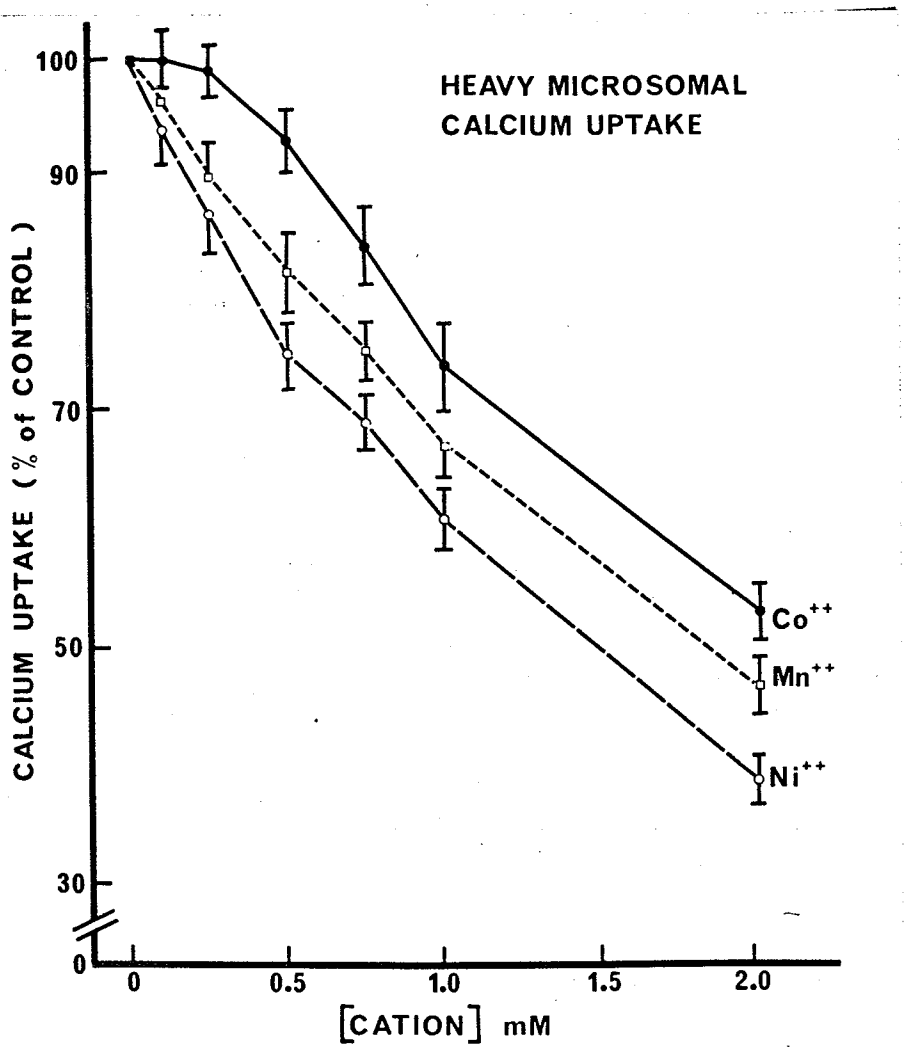



FIGURE 14 Heavy microsomal calcium uptake in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The time of incubation of the microsomes with 0.1 mM $^{45}Ca^{++}$ at 37°C was 5 min. The control value for calcium uptake was 1064 + 64 n moles/mg protein. Each value is a mean $\pm$ S.E. of 5 experiments.

TABLE XII

Time Course of Heavy Microsomal Calcium Uptake in the Absence and Presence of 1 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$

| Incubation time | Heavy microsomal calcium (nmoles/mg protein)** | | | |
|---|---|---|---|---|
| | Control | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| 30 sec | 167 ± 11.3 | 156 ± 14.0* | 154 ± 15.3* | 158 ± 15.2* |
| 1 min | 252 ± 18.6 | 212 ± 13.5 | 199 ± 16.3 | 201 ± 13.7 |
| 2 min | 400 ± 37.7 | 321 ± 18.2 | 281 ± 14.7 | 306 ± 23.4 |
| 5 min | 936 ± 24.3 | 645 ± 35.9 | 527 ± 35.3 | 626 ± 20.5 |
| 10 min | 1363 ± 67.6 | 773 ± 50.9 | 641 ± 35.2 | 952 ± 49.6 |

* Not significantly different from control ($P > 0.05$).

** The incubation medium was the same as that described for heavy microsomal calcium uptake in the Methods section. Heavy microsomes were incubated in the presence of 0.1 mM $^{45}Ca^{++}$ for various time intervals. Each value is a mean ± S.E. of 4 experiments.

TABLE XIII

Effect of 1 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on Heavy Microsomal Calcium Uptake in the Presence of Different Concentrations of Calcium

| Calcium (μM) | Heavy microsomal calcium (nmoles/mg protein)** | | | |
|---|---|---|---|---|
| | Control | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| 10 | 153 ± 11.6 | 130 ± 6.0 | 88 ± 4.3 | 118 ± 6.4 |
| 25 | 372 ± 13.3 | 309 ± 13.5 | 231 ± 12.7 | 271 ± 15.7 |
| 50 | 685 ± 35.0 | 449 ± 25.3 | 316 ± 29.0 | 391 ± 26.4 |
| 75 | 770 ± 38.0 | 591 ± 33.4 | 433 ± 25.5 | 483 ± 21.8 |

** The incubation medium for heavy microsomal calcium uptake was the same as that described in the Methods section except that various concentrations of $Ca^{++}$ were employed. All values in the presence of 1 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were significantly different from control ($P < 0.05$). Each value is a mean ± S.E. of 4 experiments.

TABLE XIV

Sarcolemmal ATP Hydrolysis in the Presence of Different Concentrations of Divalent Cations

| Concentration of cation (mM) | ATP hydrolysis (μmoles Pi/mg protein/min)* | | | | |
|---|---|---|---|---|---|
| | $Ca^{++}$ | $Mg^{++}$ | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| .10 | 2.96 ± .56 | 4.43 ± .41 | 3.97 ± .58 | 3.09 ± .58 | 2.78 ± .29 |
| .25 | 3.52 ± .20 | 5.68 ± .47 | 5.79 ± .35 | 3.60 ± .28 | 3.06 ± .25 |
| .50 | 5.74 ± .26 | 6.33 ± .39 | 8.88 ± .34 | 5.00 ± .51 | 7.38 ± .61 |
| 1.0 | 9.76 ± .40 | 9.01 ± .44 | 14.9 ± 1.3 | 5.48 ± .52 | 12.7 ± .40 |
| 2.0 | 15.1 ± 1.8 | 12.8 ± .81 | 20.5 ± 1.0 | 5.69 ± .42 | 21.1 ± 1.3 |
| 4.0 | 22.8 ± 1.4 | 21.6 ± 1.2 | 18.9 ± .95 | 9.14 ± .39 | 17.6 ± 1.1 |

* The incubation medium was the same as that described for sarcolemmal ATPase activity in the Methods section. The value for sarcolemmal ATP hydrolysis in the absence of any added cation was 1.95 ± .31 μmoles Pi/mg protein/hour. The concentration of ATP was 4 mM. Each value is a mean ± S.E. of 4 experiments.

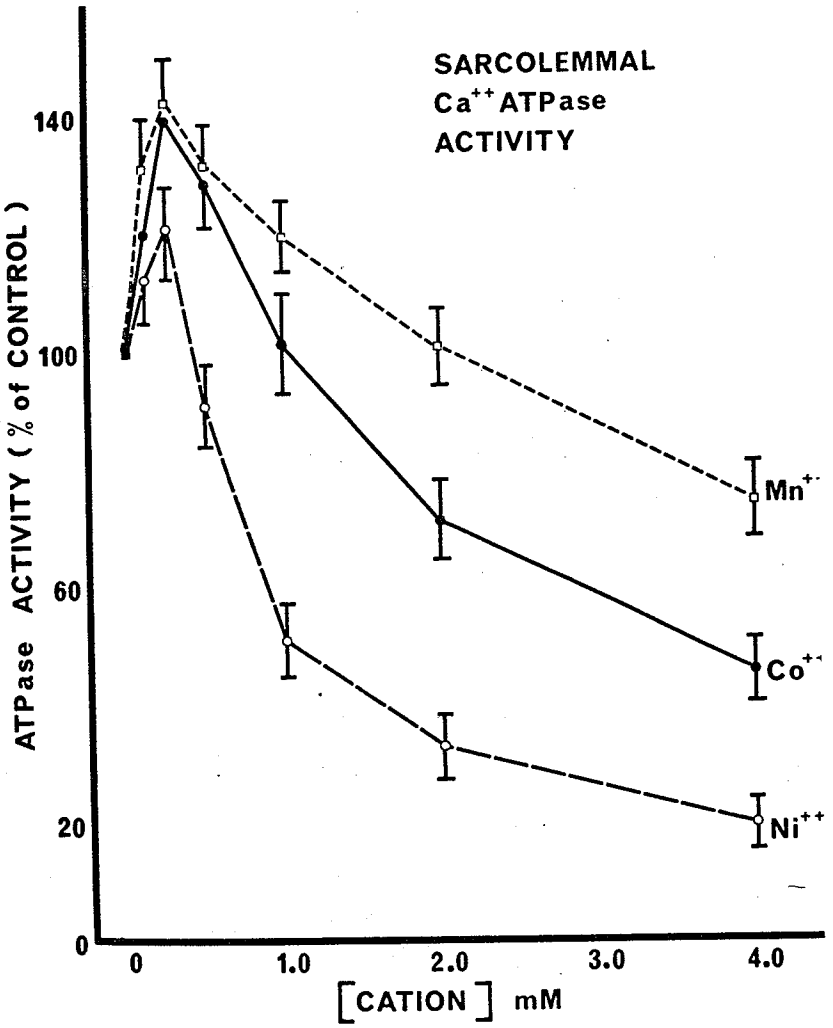
μ moles Pi/mg protein/hr respectively.

In one series of experiments, the effects of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were studied in the presence of 1.25 and 4 mM Ca ATP and the results are shown in Figures 15 and 16 respectively. The sarcolemmal $Ca^{++}$ ATPase activity in the presence of 1.25 mM $Ca^{++}$ was stimulated by 0.10 - 0.25 mM $Ni^{++}$, 0.10 - 0.50 mM $Co^{++}$ and 0.10 - 1 mM $Mn^{++}$ and depressed by 1 - 4 mM $Ni^{++}$, 2 - 4 mM $Co^{++}$ and 4 mM $Mn^{++}$. On the other hand, the $Ca^{++}$ ATPase activity in the presence of 4 mM $Ca^{++}$ was depressed significantly ($P < 0.05$) by 0.25 - 4 mM $Ni^{++}$ or $Co^{++}$ and 1 - 4 mM $Mn^{++}$. These divalent cations also produced similar effects on the sarcolemmal $Mg^{++}$ ATPase activity (Figure 17). The order of potency in decreasing $Ca^{++}/Mg^{++}$ ATPase activities were $Ni^{++} > Co^{++} > Mn^{++}$.

Sarcolemmal fraction employed in this study was found to contain ouabain sensitive $Mg^{++}$ dependent $Na^{+} - K^{+}$ ATPase activity (9.4 μ moles Pi/mg protein/hr); 80 - 90% of the $Na^{+} - K^{+}$ ATPase activity was sensitive to 2 mM ouabain. $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ in 0.1 - 4 mM concentrations were found to significantly ($P < 0.05$) decrease $Na^{+} - K^{+}$ ATPase activity (Figure 18).

In the presence of 0.1 and 1.25 mM calcium the sarcolemmal fraction was found to bind 98 and 772 n moles $Ca^{++}$/ mg protein within 5 min respectively. The effects of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on calcium binding in the presence of 0.1 and 1.25 mM calcium were examined and the results are given in Tables 15 and 16 respectively. At 0.1 mM $Ca^{++}$, 0.25 - 4 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ decreased ($P < 0.05$) calcium binding whereas at 1.25 mM $Ca^{++}$, 4 mM $Co^{++}$ or $Ni^{++}$ and 2 - 4 mM $Mn^{++}$ were effective in significantly decreasing calcium binding by sarcolemma.

The sarcolemmal adenylate cyclase activities in the absence (basal) and presence of 2 mM NaF were 124 and 517 p moles cyclic AMP/mg protein/ min. Both $Co^{++}$ and $Ni^{++}$ at 0.1 - 4 mM concentrations were found to decrease ($P < 0.05$) whereas $Mn^{++}$ at 0.1 - 4 mM concentrations was observed to increase ($P < 0.05$) the sarcolemmal basal and NaF stimulated adenylate cyclase activities (Tables 17 and 18).




F IGURE 15 Sarcolemmal $Ca^{++}$ ATPase activity in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The incubation medium was the same as that described in the Methods section except that 1.25 mM Ca ATP was used. The control value for $Ca^{++}$ ATPase was 12.1 + 0.8 μ moles Pi/mg protein/hr. Each value is a mean ± S.E. of 4 experiments.

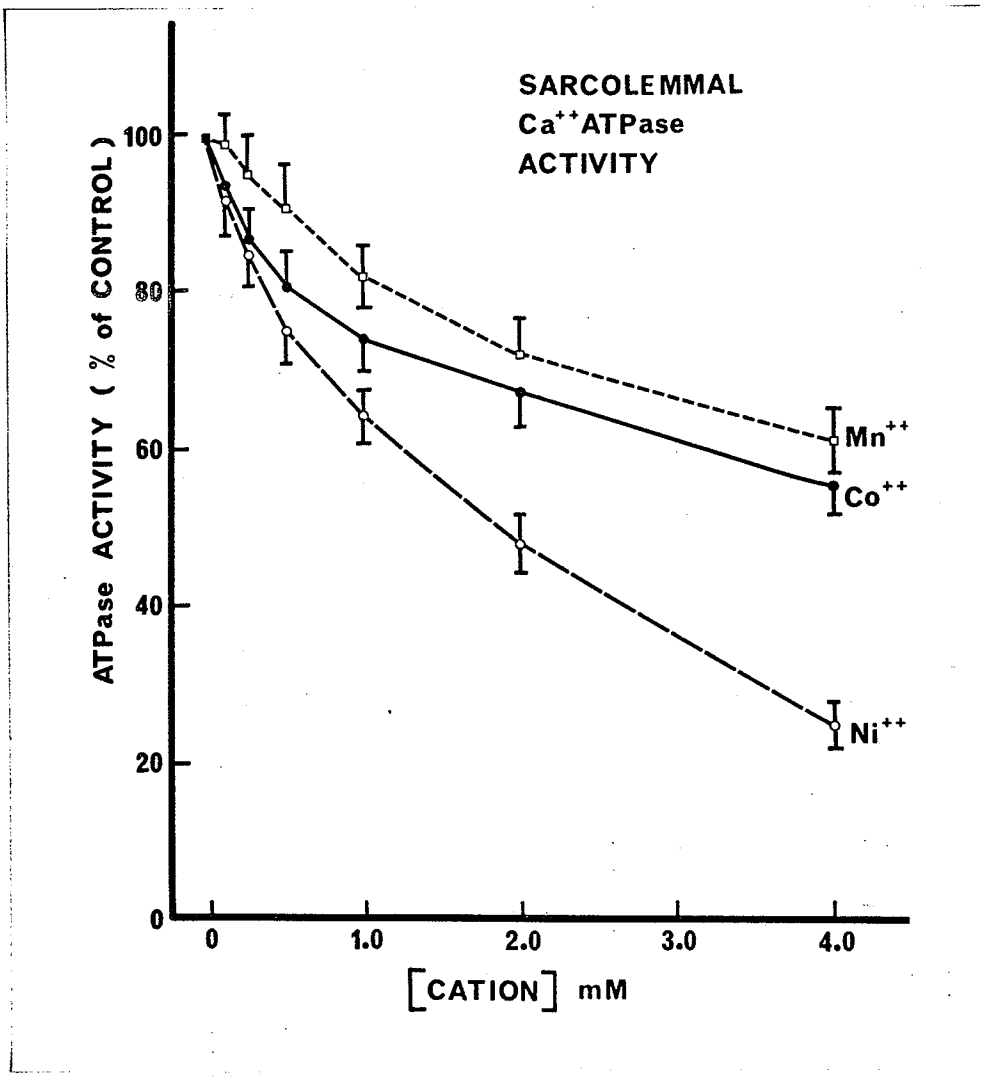



FIGURE 16

Sarcolemmal $Ca^{++}$ ATPase activity in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The incubation medium was the same as that described in the Methods section except that 4 mM Ca ATP was used. The control value for $Ca^{++}$ ATPase activity was 23.3 + 1.6 μ moles Pi/mg protein/ hr. Each value is a mean $\pm$ S.E. of 4 experiments.

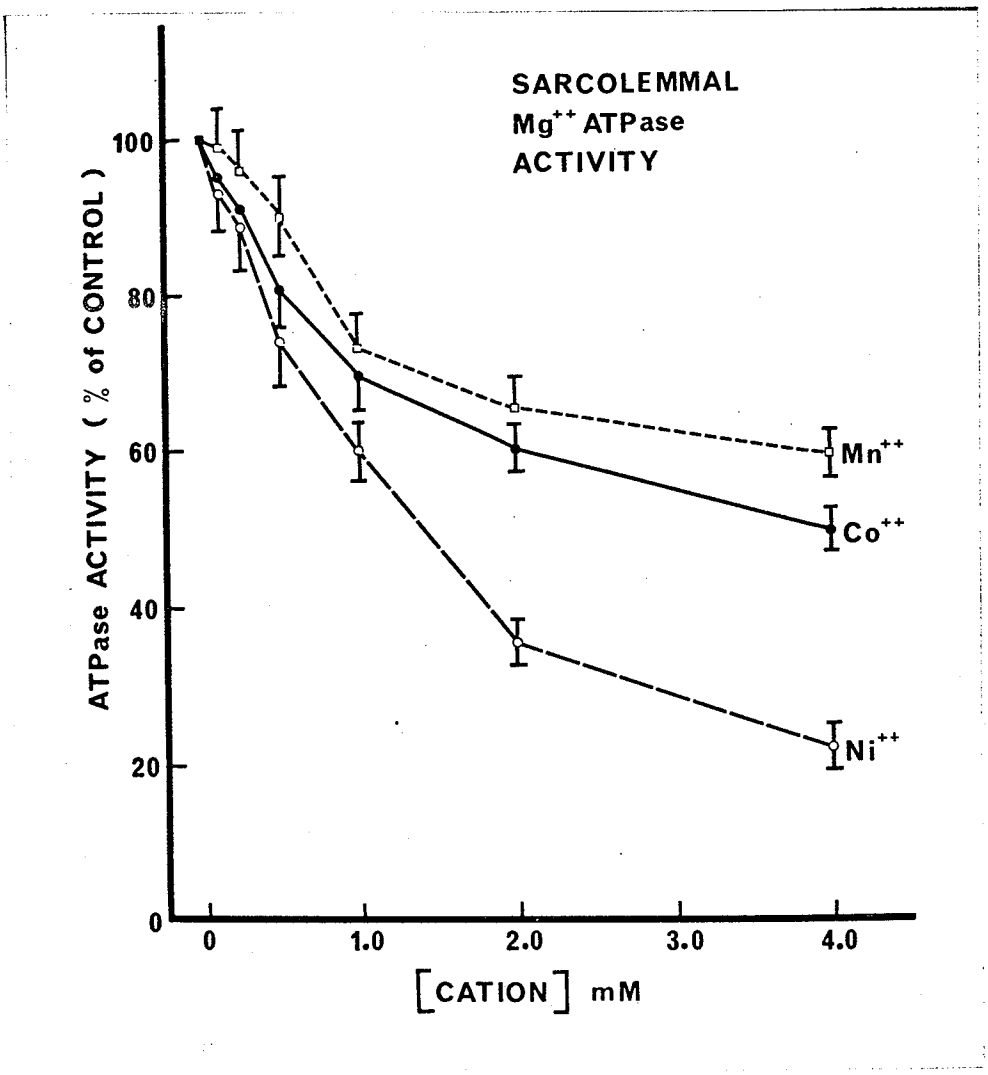



FIGURE 17

Sarcolemmal $Mg^{++}$ ATPase activity in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The incubation medium was the same as that described in the Methods section. The control value for $Mg^{++}$ ATPase activity was 22.0 + 2.5 μ moles Pi/mg protein/hr. Each value is a mean ± S.E. of 4 experiments.

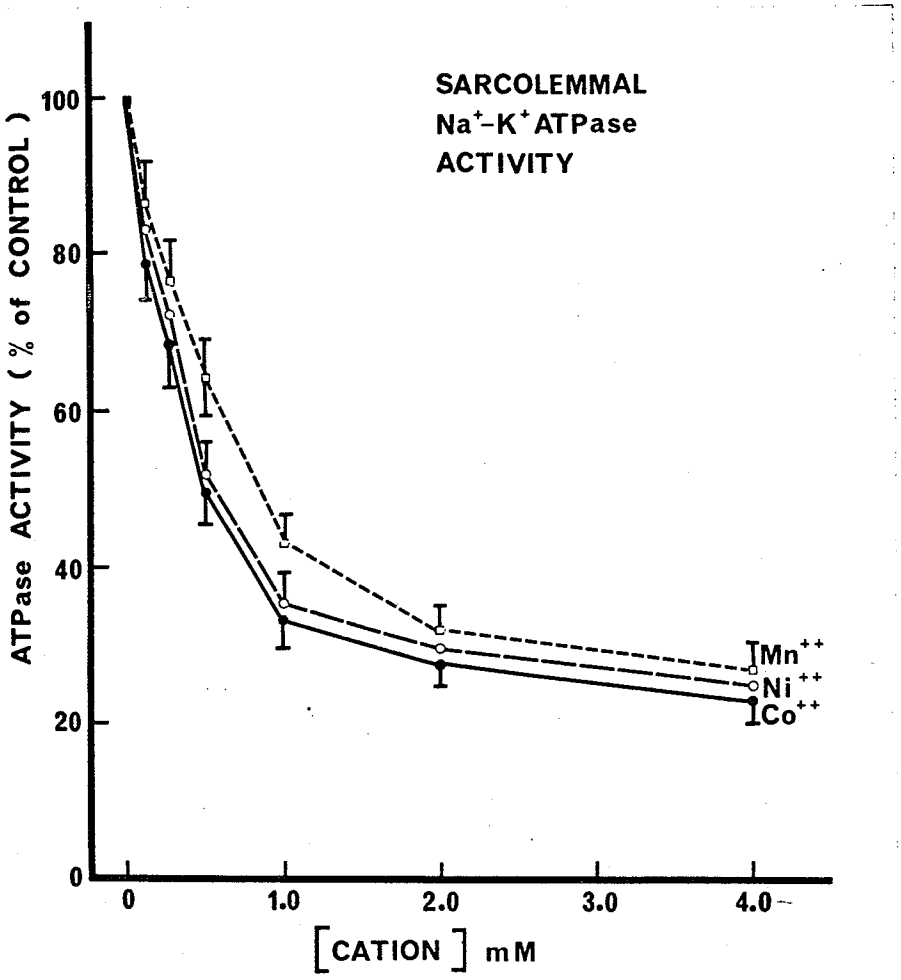



FIGURE 18 Sarcolemmal $Na^+ - K^+$ ATPase activity in the presence of different concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. The incubation medium was the same as that described in the Methods section. The control value for $Na^+ - K^+$ ATPase activity was 9.4 + 0.46 μ moles Pi/mg protein/hr. Each value is a mean $\pm$ S.E. of 4 experiments.

TABLE XV

Sarcolemmal Calcium Binding in the Absence and Presence of Different Concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$

| Concentration of cation (mM) | Sarcolemmal calcium (nmoles/mg protein)** | | |
|---|---|---|---|
| | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| 0 | 98 ± 3.2 | 95 ± 3.7 | 101 ± 6.2 |
| .10 | 96 ± 2.4* | 90 ± 2.6* | 95 ± 3.4* |
| .25 | 83 ± 3.4 | 81 ± 2.0 | 80 ± 3.3 |
| .50 | 73 ± 2.8 | 72 ± 3.1 | 73 ± 2.9 |
| 1.0 | 68 ± 2.9 | 67 ± 3.3 | 62 ± 2.5 |
| 2.0 | 63 ± 2.0 | 63 ± 2.6 | 53 ± 2.9 |
| 4.0 | 52 ± 1.7 | 51 ± 2.0 | 49 ± 2.4 |

* Not significantly different from control ($P > 0.05$).

** The incubation medium was the same as that described for sarcolemmal calcium binding in the Methods section. Sarcolemmal fractions were incubated in the presence of 0.1 mM $^{45}Ca^{++}$ for 5 minutes at 37°C. Each value is a mean ± S.E. of 4 experiments.

TABLE XVI

Sarcolemmal Calcium Binding in the Absence and Presence of Different Concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$

| Concentration of cation (mM) | Sarcolemmal calcium (nmoles/mg protein)** | | |
|---|---|---|---|
| | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| 0 | 772 ± 31 | 780 ± 27 | 776 ± 23 |
| .50 | 781 ± 22 | 783 ± 24 | 763 ± 29 |
| 1.0 | 776 ± 25 | 783 ± 18 | 756 ± 34 |
| 2.0 | 763 ± 33 | 758 ± 20 | 729 ± 23* |
| 4.0 | 717 ± 18* | 712 ± 23* | 706 ± 21* |

* Significantly different from control ($P < 0.05$).

** The incubation medium was the same as that described for sarcolemmal calcium binding in the Methods section. Sarcolemmal fractions were incubated in the presence of 1.25 mM $^{45}Ca^{++}$ for 5 minutes at 37°C. Each value is a mean ± S.E. of 4 experiments.

TABLE XVII

Sarcolemmal Basal Adenylate Cyclase Activity in the Presence of Different Concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$

| Concentration of cation (mM) | Adenylate cyclase activity* (% of control) | | |
|---|---|---|---|
| | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| .10 | 85 ± 5.8 | 77 ± 4.0 | 148 ± 7.3 |
| .25 | 77 ± 4.3 | 72 ± 3.7 | 252 ± 12.3 |
| .50 | 71 ± 3.6 | 53 ± 3.0 | 278 ± 14.6 |
| 1.0 | 61 ± 3.0 | 48 ± 2.7 | 283 ± 15.1 |
| 2.0 | 56 ± 2.7 | 34 ± 2.3 | 286 ± 17.3 |
| 4.0 | 42 ± 2.2 | 21 ± 1.7 | 308 ± 19.2 |

* The incubation medium was the same as that described for sarcolemmal adenylate cyclase activity in the Methods section. The control value for basal adenylate cyclase activity was 124 ± 4.7 pmoles cyclic AMP/mg protein/min. Each value is a mean ± S.E. of 3 experiments. All values in the presence of divalent cations were significantly different from the control ($P < 0.05$).

TABLE XVIII

Sarcolemmal NaF Stimulated Adenylate Cyclase Activity in the Presence of Different Concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$

| Concentration of cation (mM) | Adenylate cyclase activity* (% of control) | | |
|---|---|---|---|
| | $Co^{++}$ | $Ni^{++}$ | $Mn^{++}$ |
| .10 | 85 ± 4.3 | 81 ± 4.6 | 115 ± 5.1 |
| .25 | 74 ± 4.0 | 64 ± 3.2 | 135 ± 6.4 |
| .50 | 66 ± 3.2 | 51 ± 2.7 | 144 ± 6.8 |
| 1.0 | 52 ± 2.9 | 46 ± 2.6 | 178 ± 7.3 |
| 2.0 | 43 ± 2.5 | 30 ± 1.8 | 191 ± 8.6 |
| 4.0 | 34 ± 2.0 | 12 ± 1.0 | 197 ± 8.3 |

* The incubation medium was the same as that described for sarcolemmal adenylate cyclase activity in the Methods section except that 2 mM NaF was employed. The control value for adenylate cyclase activity was 517 ± 31 pmoles cyclic AMP/mg protein/min. Each value is a mean ± S.E. of 3 experiments. All values in the presence of divalent cations were significantly different from the control ($P < 0.05$).

# V. DISCUSSION

## A. Myofibrils

Myofibrils hydrolyze ATP which provides the immediate source of energy for cardiac contraction, due to the presence of $Mg^{++}$ ATPase and $Ca^{++}$ - stimulated ATPase activities. Although the role of myofibrillar $Mg^{++}$ ATPase in the generation of contractile force is not clear, myofibrillar $Ca^{++}$ - stimulated ATPase has been considered to be intimately involved in the process of myocardial contraction (165, 166). In this regard it should be noted that the level of ionized calcium, which produces half maximal myofibrillar ATPase activity has been shown to be indentical to that producing half maximal tension developement (43). In the present study we have demonstrated that $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, in 0.05 mM or higher concentrations, decrease myofibrillar $Ca^{++}$ - stimulated ATPase activity. Depression of $Ca^{++}$ - stimulated ATPase activity can be conceived to decrease the supply of energy for the sliding of action and myosin filaments and thereby result in a decline of myocardial contractility. Perfusing the hearts with a medium containing 0.1 to 1 mM concentration of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ was found to decrease contractile force developement to varying degrees. Since cardiac muscle has been shown to accumulate these divalent cations (231, 234, 241, 255), it is possible that the cardiodepressant effects of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ are partly due to their inhibitory action on the myofibrillar $Ca^{++}$ - stimulated ATPase activity.

The depressant effect of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on myofibrillar $Ca^{++}$ - stimulated ATPase may be due to their action on the actomyosin and troponin - tropomyosin systems. In this regard it should be noted that these divalent cations have been reported to bind with myosin (265). Furthermore, the inhibitory effect of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on $Ca^{++}$ - stimulated ATPase is not specific because these divalent cations in 0.05 to 0.10 mM or higher concentrations were found to inhibit myofibrillar $Mg^{++}$ ATPase activity. These cations are also capable of stimulating myofibrillar ATP hydrolysis like that of $Ca^{++}$ or $Mg^{++}$. On the other hand, troponin bound $Ca^{++}$ was found to be partially exchangeable with $Mn^{++}$ but not with $Co^{++}$ or $Ni^{++}$ (279). It was interesting to observe that $Mn^{++}$, unlike

$Co^{++}$ or $Ni^{++}$, did not produce depressant effects on myofibrillar $Ca^{++}$ - stimulated ATPase activity at 0.25 - 1 mM concentrations of Mg ATP. It thus appears that $Mn^{++}$ may affect both the calcium binding and ATP hydrolyzing sites of the myofibrils, whereas $Co^{++}$ and $Ni^{++}$ may influence the ATPase sites only. This view, however, remains to be confirmed by further studies dealing with the effects of these divalent cations on myofibrillar calcium binding. It should be pointed out here that increases in the free $Mg^{++}$ concentrations from 1 to 5 mM, in the incubation medium, has been shown to depress myofibrillar $Ca^{++}$ - stimulated ATPase activity without significantly depressing myofibrillar calcium binding (280). At any rate, the depressent effects of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ do not appear to be due to simple competition for calcium sites responsible for myofibrillar ATPase activity.

The order of potency in depressing myofibrillar $Ca^{++}$ - stimulated ATPase and $Mg^{++}$ ATPase activities was $Ni^{++} > Co^{++} > Mn^{++}$ and $Mn^{++} > Co^{++} > Ni^{++}$ respectively. On the other hand, the ability of these divalent cations to stimulate myofibrillar ATP hydrolysis, in the absence of $Mg^{++}$ or $Ca^{++}$, was not different from one another. These results indicate that $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ affect myofibrils in some complex manner. Such actions could possibly be due to differences in the abilities of these cations to form complexes with ATP as well as in their affinities for sites on myofibrils responsible for ATP hydrolysis.

It should be pointed out that the participation of the contractile proteins in the mechanisms responsible for altering myocardial contractility became apparent when Sonnenblick (281) established that changes in the contractile state of the heart is associated with changes in the activity of the contractile elements. It was proposed that the functional effects of positive and negative inotropic interventions are due to changes at the contractile sites resulting in either an increase or a decrease in the maximum rate of mechanico - chemical reactions occurring during contraction. Although the velocity of muscle shortening was correlated with myosin ATPase activity, in various types of muscle including heart (282), extensive studies of Luchi and Kritchner (283) failed to indicate any direct effect of various cardioactive agents on the cardiac myosin ATPase activity. Both negative and positive results concerning the effects of different interventions such as cardiac glycosides,

catecholamines and various cardiodepressant agents on myosin or actomyosin ATPase activities are available in the literature (116, 208). Likewise, some investigators have reported depression of myofibrillar ATPase activity in different types of failing hearts while others have failed to demonstrate such results (166, 284). Thus, direct influences of pharmacological and pathological interventions on myofibrillar ATPase activity have not been demonstrated unequivocally under in vitro conditions. This may be due to the fact that myofibrils are either resistant to these interventions or do not possess sites upon which pharmacological agents can act directly. On the other hand, different cations, including $Ca^{++}$, $Mg^{++}$, $Na^{+}$ and $K^{+}$, are known to modify the activity of isolated contractile proteins (166, 281), and it is possible that the observed effects of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ may be due to a direct interaction of these divalent cations with myofibrils. However, the exact site of their action remains to be established by employing purified preparations and a wide variety of physiochemical techniques. Whether or not the observed effects of these heavy metals on myofibrillar ATPase activities are due to their action on sulfhydryl groups (269) can not be stated on the basis of the information at hand.

B. Mitochondria

In this study we have demonstrated that mitochondrial calcium binding and uptake activities were decreased by $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. These divalent cations were also capable of stimulating mitochondrial ATP hydrolysis like $Ca^{++}$ or $Mg^{++}$ and were observed to depress mitochondrial $Mg^{++}$ ATPase activity. In addition, mitochondrial respiratory and oxidative phosphorylation activities were depressed by $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. These results indicate that these divalent cations are capable of altering heart mitochondrial function through some direct mechanism.

It is now widely accepted that the major portion of ATP in myocardial cell is generated in mitochondria through the process of oxidative phosphorylation. A depression in the mitochondrial respiratory and oxidative phosphorylation activities can be conceived to result in a reduction of the high energy phosphate stores and therefore less energy will be available for the contractile process. In this study it was observed that 0.05 - 0.10 mM concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$

decreased the mitochondrial ADP:O ratio and the RCI and increased state 4 respiration, whereas higher concentrations produced complete uncoupling of the mitochondrial respiration activities. Since 0.5 to 1 mM concentrations of these divalent cations produced a marked depressant effect upon myocardial contractility, it is likely that an impairment in the process of mitochondrial energy production, due to $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, may contribute to some extent in their negative inotropic effects. However, it should be recognized that the effects of these divalent cations on myocardial contractility are rapid in their onset and the myocardial cell is known to contain sufficient high energy phosphate stores to maintain the developement of contractile force for some period of time after inhibiting the process of energy production. Thus it is difficult to readily appreciate the role of impaired mitochondrial respiratory and oxidative phosphorylation activities due to $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ in the manifestation of acute contractile failure. At any rate, it is essential to determine the high energy phosphate stores at different degrees of depression of contractile force, due to these divalent cations, before a definitive conclusion can be drawn.

Cobalt has been reported to inhibit the oxidation of different substrates of the tricarboxylic acid cycle in liver and skeletal muscle homogenates and mitochondria due to the formation of a complex with the dithiol form of lipoic acid, a coenzyme of keto acid dehydrogenation (268). This inhibition was considered to be the key-point in the action of different divalent cations including $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on cell metabolism (269). It is possible that the observed depression in heart mitochondrial ADP:O ratio as well as RCI and increase in state 4 respiration, by $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, are due to their effect on this coenzyme. However it should be noted that $Co^{++}$ decreased the phosphorylation rate and state 3 respiration whereas $Ni^{++}$ and $Mn^{++}$ increased state 3 respiration without affecting the phosphorylation rate. Therefore, it is difficult to explain all the effects of these divalent cations on the basis of a single site of action in mitochondria. An extensive investigation on the actions of divalent cations on mitochondrial oxidative phosphorylation is obviously warranted in order to determine the exact sites of their action.

$Ni^{++}$ in 0.01 mM, or higher concentrations was found to depress

mitochondrial calcium binding and uptake activities whereas 0.10 - 0.25 mM or high concentrations of $Mn^{++}$ were required to produce an inhibitory effect. On the other hand, 0.01 mM, or higher concentrations of $Co^{++}$ decreased calcium binding and ATP - linked or ATP - succinate supported calcium uptake whereas 0.10 mM, or higher concentrations were required to inhibit pyruvate - malate supported calcium uptake by mitochondria. The reasons for these differences in concentrations of divalent cations for inhibiting mitochondrial calcium binding and uptake activities are far from clear; however, the dose response relationships reveal that the order of potency was $Ni^{++} > Co^{++} > Mn^{++}$. It is possible that such differences in the effects of these divalent cations are due to differences in their affinities for sites involved in mitochondrial calcium transport. The inhibitory effects of these cations may be independent of their effects on mitochondrial oxidation phosphorylation activity because energy - linked calcium accumulation by mitochondria has been shown to occur in preference to ATP formation (142). Furthermore, these divalent cations not only inhibited substrate - linked calcium uptake but their effects were also observed on the ATP - linked calcium binding and ATP - linked calcium uptake. Although these divalent cations inhibited mitochondrial $Mg^{++}$ ATPase activity, this effect was observed at concentrations higher than those required for inhibiting calcium transport. Since the relation of this mitochondrial ATPase with calcium transport and the mechanisms responsible for mitochondrial calcium binding and uptake are far from understood, the exact site of action of these divalent cations is a matter of speculation.

Although the contribution of mitochondrial calcium transport during cardiac contraction and relaxation cycle is controversial (157, 162), the inhibitory effects of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were observed at different intervals of incubation and at various concentrations of calcium in the incubation medium. It is of great significance that these divalent cations, particularly $Ni^{++}$ and $Co^{++}$, were capable of inhibiting calcium binding and uptake activities of mitochondria at concentrations which could be easily attained intracellularly upon perfusing the isolated heart preparations. Therefore, it is possible that the cardiodepressant effects of $Ni^{++}$ and $Co^{++}$ in particular, are mediated by their action on mitochondrial calcium

transport. It should be noted that the cardiodepressant effects of barbiturates (215) and quinidine (120, 208) have been partially explained previously on the basis of their ability to inhibit mitochondrial calcium uptake. Furthermore, depressed mitochondrial calcium accumulating ability has been reported to be associated with contractile failure in certain types of failing hearts (16, 285).

C. Sarcoplasmic Reticulum

Sarcoplasmic reticulum is considered to play a major role in the regulation of calcium movements in the cardiac cell by virtue of its remarkable ability to bind and accumulate calcium. Various pharmacological interventions have been suggested to modify myocardial contractility due to their direct or indirect inhibitory or stimulatory effects on calcium transport by the fragments (microsomal fraction) of this organelle (118, 120, 201, 211). Furthermore, a decrease in the calcium accumulating ability of the microsomal fraction has been reported for different types of failing hearts (106, 123, 124, 150, 284). Thus sarcoplasmic reticulum appears to be one of the most susceptable sites of action of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ which are known to produce a marked cardiodepressant effect. The results presented in this study indicate that the calcium uptake activity of the microsomal fraction was decreased by 0.25 - 0.5 mM concentrations of $Ni^{++}$, $Mn^{++}$ and $Co^{++}$, while their cardiodepressent effects were apparent even at 0.1 mM concentration. Thus it is unlikely that the negative inotropic effect of low concentrations of these divalent cations are mediated through their effect on the sarcoplasmic reticulum.

It should be noted that the inhibitory effects of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ were apparent at different intervals of incubation and at different concentrations of calcium in the incubation medium. On the other hand, these divalent cations were unable to affect microsomal calcium binding. This is not surprizing in view of the fact that microsomal calcium uptake, but not calcium binding, was increased and decreased or stimulated by cyclic AMP - protein kinase (117, 118) and quinidine (120, 121) respectively. Furthermore, an antibiotic ionophore, X 537A, and arsenate were reported to be more potent inhibitors of calcium uptake than calcium binding by heart microsomes (119). If it is assumed that calcium binding

and calcium uptake are two separate processes, then it can be concluded that the observed reduction in calcium uptake caused by $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ is not due to their interference at the calcium binding sites in the microsomal membrane. Since these divalent cations were found to decrease the activity of $Ca^{++}$ - stimulated ATPase, which is intimately involved in the transport of calcium across the sarcoplasmic reticular membrane (101, 103), it is likely that their inhibitory effects on calcium uptake are due to this mechanism.

The depressant effect of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on the microsomal "calcium pump" mechanism does not seem to be of a specific nature. This view is based on our finding that the microsomal $Mg^{++}$ ATPase activity was also decreased by these divalent cations. Furthermore, these cations, like that of $Ca^{++}$ and $Mg^{++}$, were capable of stimulating microsomal ATP hydrolysis. Thus it appears that the actions of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on sarcoplasmic reticular membranes are of a complex nature and their inhibitory effects on calcium uptake and ATPase activities can not be explained on the basis of simple competition with sites responsible for calcium transport. Their possible abilities to form complexes with ATP and to compete with $Mg^{++}$ can not be overlooked while explaining the basis of their action on the microsomal calcium transport process.

D. Sarcolemma

Heart sarcolemma has been shown to contain some important enzymes such as $Na^{+}$ - $K^{+}$ ATPase, adenylate cyclase, $Mg^{++}$ ATPase and $Ca^{++}$ ATPase (85, 86, 89, 100). $Na^{+}$ - $K^{+}$ ATPase, which is considered to maintain the intracellular concentrations of $Na^{+}$ and $K^{+}$ in the myocardium under certain limits, is inhibited by ouabain, a well known positive inotropic agent. On the other hand, adenylate cyclase which catalyzes the formation of cyclic AMP and thereby increases myocardial metabolism, is stimulated by catecholamines which are known to increase cardiac contractile force. Although the functional role of $Mg^{++}$ ATPase and $Ca^{++}$ ATPase activities in heart sarcolemma can not be stated with certainty, these enzymes are claimed to be involved in the movements of divalent cations across the cell membrane (82, 100). Furthermore, heart sarcolemma has been shown to possess a remarkable ability to bind a considerable amount of calcium (97, 285) and it is likely that this may serve as an important source of calcium

during the excitation - contraction process. Thus it is obvious that various agents could modify myocardial function by altering different enzymes and calcium binding activities of heart sarcolemma.

In this study we have demonstrated that $Ni^{++}$, $Co^{++}$ and $Mn^{++}$ decreased $Ca^{++}$ ATPase and $Mg^{++}$ ATPase activities, when these enzymes were assayed under optimal conditions by employing 4 mM Ca ATP or Mg ATP as substrates. The minimum concentration required for the these cations to produce a significant decrease in the enzyme activities were 0.25 - 0.5 mM for $Co^{++}$ and $Ni^{++}$ and 0.5 - 1 mM for $Mn^{++}$. It was also interesting to observe that calcium binding by sarcolemma, when studied in the presence of 0.1 mM $Ca^{++}$, was significantly decreased by 0.25 mM $Mn^{++}$, $Co^{++}$ or $Ni^{++}$. These results suggest that divalent cations interfere with certain calcium binding sites, decrease the sarcolemmal calcium stores and reduce the amount of calcium entering the myocardial cell during excitation. In this regard it should be noted that these divalent cations have been demonstrated to decrease calcium currents and have been suggested to depress myocardial contractility due to some impairment of excitation - contraction coupling process (229, 230).

In order to establish the functional significance of changes in sarcolemmal calcium binding and $Ca^{++}/Mg^{++}$ ATPase activities due to $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, the effects of these divalent cations on these parameters were studied in the presence of 1.25 mM concentration of calcium, which is known to exist in the extracellular fluid. It was observed that the calcium binding by heart sarcolemma in the presence of 1.25 mM $Ca^{++}$ was slightly but significantly inhibited by 2 mM $Mn^{++}$ and 4 mM $Co^{++}$ or $Ni^{++}$. Furthermore, low concentration of these divalent cations increased the $Ca^{++}$ ATPase activity while concentrations, 1 - 2 mM or higher, were required to inhibit the enzyme activity. It should be noted here that $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ at 0.1 to 1 mM concentrations produced varying degrees of depression in contractile force of the isolated hearts perfused with a medium containing 1.25 mM $Ca^{++}$. It is therefore difficult to readily explain the depression in contractility by 0.1 to 0.5 mM $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ on the basis of their effects on sarcolemmal calcium binding and $Ca^{++}$ ATPase activities because at these concentrations the divalent

cations had no effect on these parameters.

The results concerning the interaction of divalent cations with heart sarcolemma with respect to $Ca^{++}$ ATPase and calcium binding activities can also be interpreted to suggest that $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ do not affect the low affinity sites for calcium on sarcolemma. They may depress calcium currents through their action on the high affinity sites. This view is supported by our observation that calcium binding, in the presence of 0.1 mM $Ca^{++}$, is decreased by 0.25 - 0.5 mM concentrations of these divalent cations. The effects of these cations on $Ca^{++}$ ATPase activity, by employing low concentrations of $Ca^{++}$, could not be studied because these divalent cations were capable of stimulating ATP hydrolysis by themselves. At present it is tempting to suggest that these divalent cations may displace calcium from the high affinity sites on sarcolemma and may enter the myocardial cell upon depolarization and thereby acting as "false couplers" in the place of calcium in the excitation - contraction coupling mechanism. In this regard it should be noted that a $Mn^{++}$ action potential has been reported to occur in cardiac muscle (235). Furthermore, calcium - exchange studies have indicated that these divalent cations release calcium from a small membrane - located component (242). In addition, increasing the concentration of extracellular calcium has been shown to overcome the cardiodepressant effect of $Mn^{++}$ (239).

Inhibition of the sarcolemmal $Na^{+}$ - $K^{+}$ ATPase activity by $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ would tend to increase myocardial contractility by making more calcium available, through direct or indirect mechanisms similar to those proposed for cardiac glycosides (90). On the contrary, concentrations of these divalent cations, which inhibited $Na^{+}$ - $K^{+}$ ATPase activity, were found to depress myocardial contractility. If it is assumed that these divalent cations displace membrane bound calcium, then less calcium would be available from sources associated with $Na^{+}$ - $K^{+}$ ATPase inhibition. The inhibition of $Na^{+}$ - $K^{+}$ ATPase would increase the movements of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ into the cell in place of calcium. Since these divalent cations can not substitute for calcium in initiating events leading to contraction, no positive inotropic action like that observed with the cardiac glycosides would be apparent. This view is supported by

the observation that $Mn^{++}$ antagonizes the positive inotropic effect of ouabain and its ability to increase calcium exchange (239). Furthermore, ouabain has also been reported to partially antagonize the effect of $Ni^{++}$. A detailed study on the interaction of $Na^{+}$ - $K^{+}$ ATPase and these divalent cations is warranted to gain insight into this mechanism.

The basal and NaF - stimulated activities of sarcolemmal adenylate cyclase, which is known to regulate cellular permeability, were found to be inhibited by $Co^{++}$ and $Ni^{++}$, whereas $Mn^{++}$ was observed to have a stimulatory effect. Depression in the adenylate cyclase activity by $Co^{++}$ and $Ni^{++}$ would lead to less formation of cyclic AMP and decrease calcium movements into the myocardium since cyclic AMP has been implicated in the regulation of calcium influx (89). On the other hand, stimulation of adenylate cyclase by $Mn^{++}$ would increase the movements of calcium in the myocardial cell and thereby a positive inotropic response should be evoked but this mechanism may not be effective if $Mn^{++}$ displaced $Ca^{++}$ at the sarcolemmal sites. At any rate, the opposing effects of $Mn^{++}$ and $Co^{++}$ or $Ni^{++}$ on the sarcolemmal adenylate cyclase may contribute in causing some differences in the degree of contractile failure. The results reported here indicate that both $Co^{++}$ and $Ni^{++}$ at 1 mM concentrations rapidly abolished the ability of the heart to generate contractile force whereas 1 mM $Mn^{++}$ caused only a gradual but marked depression in contractility.

E. Intergrated Mechanism of Action of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$

From the foregoing discussion of the results presented in this study, it is clear that divalent cations such as $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ may exert their cardiodepressant action through complex mechanisms involving sarcolemma, sarcoplasmic reticulum, mitochondria and myofibrils. Electron microscopic examination of hearts perfused with these divalent cations revealed that their negative inotropic effect was not due to any alteration in the ultrastructure of myocardium. However, dose-response relationship measuring different parameters revealed that mitochondrial oxidative phosphorylation and myofibrillar ATPase activities were depressed by concentrations (0.05 mM) of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ which could be easily attained intracellularly upon perfusing the myocardium with 0.5 to 1 mM concentrations of

these divalent cations. We believe that an impairment in the process of energy utilization may play an important role in eliciting acute contractile failure whereas a defect in the energy production may be of importance in the long term effects of these divalent cations.

In this study, it was observed that mitochondrial calcium transport was depressed by relatively low concentrations (0.01 mM) of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, whereas microsomal calcium accumulation was affected at 0.25 - 0.5 mM concentrations. Although these results indicate that changes in mitochondrial calcium transport may play a predominent role in acute contractile failure due to low doses of divalent cations, changes in microsomal calcium uptake may be of some relevence for long term myocardial effects with high doses of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$. At any rate, depression in the calcium accumulating abilities of subcellular organelles can be considered to result in decreased calcium content thereby making less calcium available for release upon excitation of myocardium. This mechanism could explain the cardiodepressant effect of divalent cations depending upon their concentrations in the perfusion medium. Decreased abilities of the subcellular organelles to accumulate calcium could raise the cytoplasmic level of calcium and this may be responsible for the increased resting tension seen in certain experimental conditions.

Stimulation of adenylate cyclase by $Mn^{++}$ would favour increasing the amount of $Ca^{++}$ or $Mn^{++}$ entering the cell; but $Co^{++}$ and $Ni^{++}$ were found to inhibit this system. It was most significant to observe that concentrations of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ which decreased contractile force also inhibited the $Na^{+} - K^{+}$ ATPase activity. Since inhibition of sarcolemmal $Na^{+} - K^{+}$ ATPase is considered to increase the movements of calcium into the myocardial cell, it is likely that the movements of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ are also acelerated due to this mechanism. These cations upon entering the cell may serve as "false - couplers" due to their inability to substitute for calcium in the events leading from excitation to contraction. At any rate, sufficient calcium would not be available for entrance into the myocardial cell because these divalent cations may displace calcium from the sarcolemmal sites. Decreased calcium binding in the presence of $Co^{++}$, $Ni^{++}$ and

$Mn^{++}$ would reduce the sarcolemmal calcium contents and thus make less available for release upon excitation. Inhibition of $Ca^{++}/Mg^{++}$ ATPase, which may regulate calcium channels in some unknown manner, may also contribute in reducing the amount of calcium entering the myocardial cell depending upon the concentration of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$ in the medium. Therefore, the actions of these divalent cations on sarcolemma may play a crucial role in the manifestation of their depressant effects on myocardial contractility.

It should be recognized that the sensitivities of various systems, to $Co^{++}$, $Ni^{++}$ or $Mn^{++}$, were different from each other and these divalent cations exerted varying degrees of action on each parameter examined in this study. This could be interpreted to mean that the contribution of each component to the overall effect of one cation may differ from that of the other, depending upon the concentration employed for eliciting the effect. In spite of many similarities among the mechanisms of actions of $Co^{++}$, $Ni^{++}$ and $Mn^{++}$, some differences are quite obvious. On the basis of information at hand, it can be concluded that the cardiodepressant effects of these divalent cations are elicited through complex mechanisms involving different cellular components, and it is highly probable that these cations cause intracellular calcium deficiency in addition to serving as "false couplers" in the excitation - contraction coupling mechanism.

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