PROPERTIES OF MEMBRANE BOUND ENZYMES IN NORMAL AND FAILING HEARTS

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To my mother and father

with great love and affection

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ABSTRACT

Heart sarcolemmal fraction was isolated from the heart homogenate by hypotonic shock and 0.4 M LiBr treatments. The time required for the isolation of this fraction was about 5 h. This membranous fraction from the dog heart contained $Na^+ - K^+$ stimulated ATPase, Mg^{++}ATPase, Ca^{++}ATPase and adenylate cyclase. The specific activities of $Na^+ - K^+$ATPase (10 μmoles Pi/ mg per h) and adenylate cyclase (280 pmoles cyclic AMP/mg per min) in the membrane fraction were 7 - 8 fold of those in the heart homogenate. About 90% of the total $Na^+ - K^+$ATPase or adenylate cyclase activies of the heart homogenate were recovered in the membrane fraction. The membrane fraction consisted of empty sacs of varying shapes and sizes and was devoid of most of the mitochondrial, microsomal and myofibril contaminants as evidenced by electron microscopic, marker enzymes and ATP supported Ca^{++} binding studies. In view of the time required for the isolation of the membrane fraction as well as the activities of different membrane bound enzymes, no attempt was made to further purify this preparation for the purpose of this study.

The properties of $Na^+ - K^+$ATPase in the dog heart membrane fraction were compared with those of the NaI treated membrane fraction and the solubilized enzyme preparation. The enzyme activities in the membrane fractions with or without NaI treatment were markedly lower than that of the solubilized preparation. The K_m values for the $Na^+ - K^+$ATPase of the membrane fraction, NaI treated membrane fraction and solubilized enzyme preparation were 0.75, 0.70 and 0.31 mM (MgATP) respectively. The mean values for the concentrations of K^+ varied from 1.2 - 2.7 mM and those for Na^+ varied from 12 - 20 mM for half maximal

activation of $Na^+ - K^+$ATPase in these preparations. The mean values for the concentrations of ouabain varied from 2.4 - 3.2 μM and those for calcium varied from 0.5 - 1.2 mM for 50% inhibition of $Na^+ - K^+$ATPase in these preparations. The pH optimum for $Na^+ - K^+$ATPase activity in all of the three preparations was about 7.5. The inhibitory effects of both Ca^{++} and ouabain were found to be pH dependent. Different monovalent cations such as Rb^+, NH^+_4, Cs^+, Li^+ and $choline^+$ were poor substitutes for K^+ in activating the $Na^+ - K^+$ATPase in these preparations. Since the enzyme activity curves on varying the concentration of MgATP, K^+ or Na^+ were sigmoidal in shape and the n values obtained by Hill plots were greater than one, this evidence is considered to extend support for the allosteric nature of the cardiac $Na^+ - K^+$ ATPase.

The mean K_m values were 0.90 and 0.95 mM and those for V_{max} were 17.2 and 16.0 μmoles Pi/mg per h for the dog heart membrane Ca^{++}ATPase and Mg^{++}ATPase respectively. Other divalent cations such as Mn^{++}, Co^{++} and Ni^{++} were also found to stimulate ATP hydrolysis in this fraction. Excess of ATP was inhibitory to the ATP hydrolysis due to various divalent cations. Ni^{++}, Co^{++}, Mg^{++} and Mn^{++} were shown to depress the ATP hydrolysis due to Ca^{++}. The Ca^{++}ATPase activity in the dog heart membranes was also inhibited by Na^+ whereas K^+ had no effect.

The adenylate cyclase activity of the dog heart membranes was increased by about 35% and 4 fold by epinephrine and NaF respectively. The adenylate cyclase activity in the absence or presence of these agents showed a broad

pH optimum between 8.0 and 8.5. Both NaF and epinephrine increased the enzyme activity at all the concentrations of Mg^{++} employed in this study. These agents increased V_{max} (286 pmoles cyclic AMP/mg per min) without any changes in the K_m value (0.82 mM) for ATP.

Although other catecholamines such as norepinephrine and isoproterenol increased adenylate cyclase activity, various sympathomimetic agents such as tyramine, metaraminol, ephedrine and nicotine were ineffective. The activation of adenylate cyclase due to epinephrine was blocked by a well known β - adrenergic blocking agent, propranolol. None of the above sympathomimetic agents or cyclic AMP were found to affect the Mg^{++}ATPase, Ca^{++}ATPase or Na^{+}- K^{+}ATPase activity of the dog heart membranes.

Perfusing the rat hearts with Na^{+} free or K^{+} free medium decreased Na^{+}- K^{+}ATPase activity without affecting the activities of adenylate cyclase, Mg^{++}ATPase or Ca^{++}ATPase. The activities of all the membrane bound enzymes did not change on perfusing the hearts with Mg^{++} free medium. When the hearts were perfused with Ca^{++} free medium, the activities of adenylate cyclase, Mg^{++}ATPase and Na^{+}- K^{+}ATPase were decreased without any changes in the Ca^{++}ATPase activity. The stimulatory action of NaF on the adenylate cyclase was enhanced on perfusing the hearts with Ca^{++} free medium. The membrane bound enzyme activities of the hearts preperfused with Ca^{++} free medium were further depressed on perfusion with medium containing normal amounts of calcium. These results suggest the role of extracellular calcium in membrane function.

The activities of Ca^{++}ATPase, Mg^{++}ATPase and Na^{+} - K^{+}ATPase in the membrane fraction obtained from the failing hearts of genetically cardiomyopathic hamsters (UM-X7.1) with advanced degree of congestive heart failure were lower in comparison to the values of the preparations from the normal healthy animals. Although the basal adenylate cyclase activity of the failing heart membranes was not different from the control, the adenylate cyclase activating responses to both NaF and epinephrine were depressed. These results substantiate the possibility of membrane abnormality in heart failure.

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INTRODUCTION AND STATEMENT OF THE PROBLEM

Although adequate cardiac performance is considered to depend upon proper function of the contractile unit, it is becoming clear that various membranous systems, such as mitochondria, sarcoplasmic reticulum and the cell membrane (sarcolemma) are intimately involved in its regulation. Such a regulatory role of these membranes is mainly attributed to their properties of maintaining the cytoplasmic concentrations of various ions at a desired level. Over the past decade a considerable amount of work has been conducted concerning the roles of mitochondria and sarcoplasmic reticulum in heart function and metabolism in normal and diseased states[1 - 12], however, relatively little attention has been focused upon the cell membrane. This has been mainly due to difficulties in isolating cardiac sarcolemma in a relatively pure form. It was therefore considered desirable to isolate heart sarcolemma for the purpose of studying its properties.

Sarcolemma is believed to be the primary site of localization of important enzymes such as adenylate cyclase, Na^{+} - K^{+}ATPase, Mg^{++}ATPase and Ca^{++} ATPase[4, 13 - 19]. These enzymes are considered to play an important role in heart function and metabolism by regulating ion movements across the cell membrane as well as providing signals for various metabolic processes. Therefore, monitoring the activities of these enzymes has been thought to provide information concerning the status of the heart membrane which in turn is reflected in cardiac function and metabolism. It was, therefore, the purpose of this study to determine the activities of these enzymes under various experimental conditions in preparations

obtained from normal dog hearts. Since various sympathomimetic agents are known to augment myocardial contractility and metabolism[20, 21], it was decided to examine the effects of some of these agents on the activities of the membrane bound enzymes. The properties of $Na^+ - K^+$ ATPase in the membrane preparations were compared with those in the solubilized enzyme in order to gain further information on cardiac $Na^+ - K^+$ pump mechanisms.

Since different cations such as Mg^{++}, Na^+, K^+ and particularly Ca^{++} are known to play a crucial role in the control of myocardial contractility[22 - 26], it was decided to examine the changes in activities of Mg^{++} ATPase, Ca^{++} ATPase, $Na^+ - K^+$ ATPase and adenylate cyclase of the membrane fractions obtained from isolated rat hearts perfused with media in the absence of Mg^{++}, Na^+, K^+ or Ca^{++}. In view of the crucial role played by Ca^{++} in the process of excitation-contraction coupling, metabolic stimulation and maintenance of membrane integrity, a detailed investigation concerning the changes in enzyme activities was undertaken on preparations obtained from hearts perfused with Ca^{++} free medium.

On the basis of studies concerning calcium transport by subcellular particles, adenylate cyclase activity of the washed cell particles, and $Na^+ - K^+$ ATPase activity of the solubilized enzyme preparation[12, 27 - 29], it has been claimed that the cell membrane is abnormal in failing hearts. An attempt was therefore made to find out if alterations in the activities of $Na^+ - K^+$ ATPase, Mg^{++} ATPase, Ca^{++} ATPase and adenylate cyclase occur in membrane preparations obtained from cardiomyopathic hamsters (UM-X7.1) with an advanced degree of heart failure. This new line of hamsters has been developed with 100% incidence of congestive heart failure[30].

REVIEW OF THE LITERATURE

1. General Properties of Excitable Membranes

Although the cell membrane was thought of as merely supportive or protective, there is now much evidence that it contains highly specific and sensitive receptors for chemical or hormonal signals[31]. Various excellent reviews concerning the membrane molecular organization, ultrastructure, bioelectric properties and transport have appeared in the literature[32 - 35]. The plasma membrane constitutes the real permeability barrier of the cell and gives the membrane its high electrical resistance and capacitance. The most accepted molecular model of the structure of plasma membranes is that first proposed by Danielli and Davson[36] and later refined by Robertson[37] as the unit-membrane hypothesis. It proposes that the lipids of the membrane, which comprise some 40 - 50% of its mass, are arranged in a bilayer, with the hydrocarbon chains of the two lipid layers apposed to form a continuous, nonpolar hydrocarbon phase. On either side are monolayers of protein, which comprise some 50 - 60% of the membrane mass.

The unit membrane hypothesis was originally postulated to account for the basic structure of all types of membranes; however, it is now apparent that different membranes may vary substantially in molecular composition (particularly of the lipids), enzymatic activity, transport functions, thickness, and in the type of image yielded by high-resolution electron microscopic techniques, such as negative contrast and freeze-etching[32, 38 - 40]. Furthermore, the plasma membrane of any given cell type is not necessarily uniform over its whole surface

and may be locally differentiated at desmosomes, tight junctions, and synapses, and may indeed possess a microscopic non-uniformity as in a two dimensional molecular mosaic. In addition, membranes may also change locally in structure as a function of activity. There is, however, one feature of the unit membrane structure, namely the lipid bilayer, which still accounts best for the characteristic permeability and electrical properties of plasma membranes.

Each type of membrane contains a characteristic set of complex lipids in a specific molar ratio, which appears to be genetically determined. Each of the polar or amphipathic phosphoglycerides of membranes contains a polar "head" and two non-polar hydrocarbon "tails" contributed by C_{16}- C_{20} fatty acids or aldehydes, which are about 20 Å long when fully extended. The polar heads of the different membrane lipids differ quite significantly in size, conformation, and electrical charge. Just as the side chains or R groups of the 20 different amino acids of proteins are known now to be determinants of the three dimensional structure of proteins, the characteristic polar heads of the 15 or 20 different types of polar lipids found in most membranes may similarly be determinants of membrane properties. Presumably each type of lipid may contribute some specific characteristic or property to the membrane. Most plasma membranes also contain considerable cholesterol, which forms hydrophobically stabilized complexes with those phosphoglycerides having unsaturated fatty acids. In such complexes the fatty acid tails of the phosphoglycerides become immobilized and rigid. Plasma membranes, because of their relatively high cholesterol content, are more rigidly structured and "tighter" than other types of membranes[41].

One of the strongest pieces of evidence in support of the lipid bilayer model is the fact that, in the complete absence of protein, phospholipids in aqueous systems spontaneously form bilayers in the form of flat micelles as large closed vesicles. Most pertient are the studies by Mueller and Rudin[42] and by Maddy and co-workers[43], who have shown that phospholipid bilayers may be formed in apertures separating two aqueous phases. Such bilayers have low permeability to polar solutes, high permeability to water and extremely high electrical resistance and capacitance comparable to that of the plasma membrane. Such lipid bilayers can also be made electrically excitable in the presence of an ion gradient[44] and these have therefore become extremely important models of natural membranes.

Little is known of the molecular structure of uncontaminated membrane protein; however, research on the structure protein of other membranes[45, 46] suggests that the monomeric form may have a molecular weight in the range of 20,000 - 50,000 and that the monomers readily associate with each other to form insoluble aggregates stabilized by hydrophobic interactions. Optical rotatory dispersion and circular dichroism spectra of the proteins of different types of plasma membranes are very similar, which suggests that the peptide chains of membrane proteins are folded in a characteristic manner and that they may contain some α -helical characteristics. Recent work also suggests that there are several, and possibly many, different molecular species of structural proteins in a given membrane. Membrane structure proteins are undoubtedly genetically coded and they may in turn code or specify the specific content and ratio of the various

lipids of the membrane. One hypothesis[47] suggests that each species of membrane protein may be able to bind selectively a single type of membrane phospholipid. Thus, a two dimensional array of different species of membrane structure proteins might code a two dimensional mosaic of specifically bound lipids.

Nuclear magnetic resonance measurements have revealed that the proteins are relatively fixed in the membrane structure but that the lipids have considerable freedom of movement[48, 49]. The polar heads of the lipids behave as thought they are in a dilute aqueous environment and they are at least partially susceptible to enzymatic attack by phospholipases[50]. The hydrocarbon tails are relatively free to move, although as noted above, they are much more rigid and fixed in those membranes having a high cholesterol content.

For many years it has been thought that movements of Na^+ and K^+ are specific elements in the excitation of the cell and the generation of the action potential, particularly since the classical work of Hodgkin[51] showed that the sum of the electrical currents carried by the entry of Na^+ and the exit of K^+ can be quantitatively related to the transmembrane potential and the transmembrane conductance at all points in the action potential. Specifically, it has been assumed that the first result of stimulation is the entry of Na^+. However, more recent experiments by Tasaki and his colleagues[52] are opening a new chapter in our understanding of the role of cations in the excitation process. The most surprising finding in their work is that neither Na^+ or K^+ is required on the inside or outside of the membrane to support excitability. Phosphate salts of the univalent cations Li^+, Rb^+ or Cs^+, or substituted ammonium ions, can

maintain excitability of cells and show an action potential if they are externally bathed with divalent cations such as Ca^{++}, Ba^{++} or Sr^{++}, despite the completely "unphysiological" nature of the internal and external ions. Na^{+} or K^{+} are thus not specific requirements for exciting the membrane. Tasaki and his colleagues[52] have concluded that their data are not in accord with any hypothesis for the excitation process that requires as the first event a specific increase in Na^{+} permeability and influx of Na^{+} into the cell. It is therefore now proposed that in the resting state the outer region of the cell membrane contains bound divalent cations, probably Ca^{++} derived from the external medium. On stimulation by an outward directed current, some of the bound Ca^{++} is replaced by univalent cations derived from the internal medium (K^{+} in normal cell). This ion exchange process is suggested to trigger a change in the conformation of membrane subunits to a second state in which membrane conductance is increased permitting Na^{+} to diffuse in and K^{+} to diffuse out. At the end of the action potential the membrane bound K^{+} is displaced by Ca^{++} again, returning it to its original resting state of conformation. It is possible that the Ca^{++} binding sites, which are small in number, may be provided by specific sialic acid residues of the membrane gangliosides. Actually, only a very small fraction of the membrane area needs to undergo loss of Ca^{++} to cause its all-or-none excitation[52].

Whatever events may determine the process of excitation, two properties of the excitable cell membrane suggest that it undergoes co-operative transitions. One is the finding that interaction of only a few molecular sites in the membrane

is required to trigger a change of the whole membrane. The other is the all-or-none nature of the response of the excitable cell to increasing stimulus intensity. These properties are suggestive of the behavior of certain solid-state systems[53] and of the behavior of allosteric or regulatory proteins and enzymes[54, 55]. At least some investigators[52, 56, 57] have postulated that electrical or chemical perturbation of a few specific membrane subunits causes a change in their conformation and that this change in conformation is then physically transmitted to neighboring subunits in a co-operative manner. The membrane subunits are considered to exist in two states: the resting and the excited states. The free energy difference between these two states cannot be large, since only a very small energy input is sufficient to trigger the change. Presumably there is a phase transition, or a transition between two metastable states. In principle, the membrane can be visualized in the same terms as the hemoglobin molecule when it undergoes oxygenation with its classical sigmoid dependence on oxygen partial pressure. In the excitable membrane the sigmoidicity is of a much higher order, so that it shows all-or-none and amplifcation characteristics.

One of the most significant contributions in membrane biology was the report by Mueller and Rudin[44] that the macrocytic antibiotic alamethicin, which is a charged circular molecule, can impart electrical excitability to a synthetic phospholipid bilayer in an aperture separating two aqueous phases having different concentrations of K^+. Such an artificial membrane shows an action potential on electrical stimulation and an increased cation conductance. These synthetic bilayers containing alamethicin were found to simulate the electrical behavior

of a wide variety of natural membranes. Mueller and Rudin[44] have postulated that a group of five or six alamethicin molecules, each binding a K^+ ion, may aggregate into a stack which may extend through the membrane. Normally, in the resting state, this stack does not allow the free passage of K^+. However, when the membrane is perturbed by the electrical stimulus, one of the charged alamethicin subunits is assumed to undergo conformational change, and results in its directional opening. This rationalization of the action of alamethicin suggests that a similar principle underlies the operation of the normal Na^+ and K^+ gates of the membrane. These gates may be comprised of analogous stacks of circular Na^+ binding and K^+ binding molecules which are normally closed and do not allow Na^+ and K^+ to pass through when the membrane is in the resting state. When stimulated, they change conformation and allow unidirectional passage of Na^+ and K^+ down their gradients thus producing an action potential.

Depolarization is usually associated with an increase in membrane permeability and there seems to be general agreement that the cellular permeability is regulated by certain enzymes such as Na^+ - K^+ATPase, Ca^{++}ATPase, Mg^{++}ATPase and adenylate cyclase which are bound to the cell membrane. For example, Na^+ - K^+ATPase has been implicated in the active transport of Na^+ and K^+ across the cell membrane[17, 18]. The presence of Ca^{++}ATPase and Mg^{++}ATPase has been demonstrated in membranes isolated from various tissues[19, 58, 59] and these enzymes can be presumed to be involved in the transport of Ca^{++} and Mg^{++} respectively. Adenylate cyclase, an enzyme which catalyzes the formation of adenosine 3', 5'-monophosphate (cyclic AMP), has been speculated to regulate

the cell permeability in addition to its well established role in cellular metabolic events such as glycolysis and lipolysis[13 - 16]. Since membrane integrity and particularly the lipoprotein composition of the membrane is essential for the full expression of the activities of membrane bound enzymes, we feel that the status of membrane function can be assessed by monitoring the activities of adenylate cyclase, Mg^{++}ATPase, Ca^{++}ATPase and Na^{+} - K^{+} ATPase.

2. Studies on Myocardial Cell Membrane (Sarcolemma)

Although considerable information is available on the electrical behavior of the heart cell membrane, very little is yet known of its molecular composition and structure because of the difficulties in obtaining sufficient material uncontaminated by the intracellular membranes such as mitochondria and sarcoplasmic reticulum for biochemical studies. The sarcolemma has two components: a thin, electron dense plasma membrane and a much thicker amphorus coating, basement membrane which surrounds the entire cell and is its outermost layer. The basement membrane is a mucopolysaccharide and has a multiplicity of negative charges by which it can bind cations. The sarcolemma sends penetrating finger-like invaginations into the myocardial cell, thus creating an intricate pattern of tubules which are known as the transverse tubular system (T-system). Both the basement membrane and the plasma membrane participate in the formation of the T-system. The tubules come into intimate contact with the lateral sacs of the sarcoplasmic reticulum, the main site of calcium storage.

It is generally believed that the excitatory impulse along the sarcolemma travels down the T-system and promptly releases calcium ion from the lateral sac of the sarcoplasmic reticulum in the vicinity of the myofibrils and thus initiates contraction. Since myocardial cells such as the atrial and Purkinje cells[60], as well as the ventricular cells of embryonic or neonatal chickens have no T-system[61], it appears that the T-system is not essential for the rapid propagation of excitation throughtout the cell or for the coupling of excitation to contraction. It should be noted that the time between excitation and the beginning of contraction is about 20 ms in cardiac muscle. Thus it is not necessary that the exciting impulse should release calcium from the sarcoplasmic reticulum almost simultaneously at the level of each sarcomere, as thought previously. On the other hand, current evidence points to the sarcolemmal basement membrane as the source of excitation-contraction coupling in the myofiber[62]. Accordingly, the calcium which is presented to the area of the contractile filaments is released from the superficial cell membrane and travels inwards to the area of the sarcomeres by a simple process of diffusion. Calcium ion diffuses in myoplasm at a rate of 1 μ/ms and would take 5 ms in order to reach the contractile unit of the cardiac cell with an average diameter of 10 μ. Whether or not sarcolemmal calcium is sufficient for the purpose of contraction is not clear at present. The role of extracellular calcium in this regard cannot be ignored.

The myocardial cell is capable of excitation and can spontaneously depolarize in a periodic fashion. The process of excitation and depolarization depends upon the properties of the sarcolemma, the T-system and the intercalated

disc. The excitation is coupled to contraction so that the cell shortens in response to stimulation. One of the most important areas currently under investigation in cardiac cell physiology is the mechanism by which excitation is coupled to contraction in the myofiber. There is growing evidence that Ca^{++} enters the myocardium during the plateau of the cardiac action potential[63, 64] and augmentation of this Ca^{++} influx can increase myocardial contractility[65, 66]. Some cations such as Ni^{++} and Co^{++} have been found to reduce or even completely suppress the myocardial contractility, while the height and the shape of the action potential remain nearly unchanged[67]. On the other hand, Mn^{++} ions are known to have similar effects on cardiac contractility without any pronounced decrease in the plateau of the action potential[68, 69]. Procaine, an agent which blocks the movement of Ca^{++} through membranes, prevented the rise in exchangeable Ca^{++} by depolarization while leaving the depolarization unchanged[70]. These observations lend support to the general concept that Ca^{++} ion is not only essential for the contractile events but also plays a crucial role in membrane events associated with excitation and depolarization of the cardiac cell.

Several investigators have attempted to isolate heart sarcolemma for biochemical, pharmacological and pathological studies. Stam et al.[71] were the first to report the isolation of myocardial sarcolemma, after extraction with NaI or KI. Morphologically, the preparation progressed from fibers to fiber sized, membrane enclosed striation free sacs. This preparation contained both Mg^{++}ATPase and Na^{+} - K^{+}ATPase (7 μmoles Pi/mg per h) activities and

was found to be associated with phospholipases A and A_2 as well as lysophospholipase[72]. In addition, this preparation has been found to contain Mg^{++} activated, Ca^{++} stimulated, ouabain sensitive ATPase[73]. Other workers, however, have demonstrated the presence of Mg^{++} independent, Ca^{++} stimulated, ouabain insensitive ATPase in heart sarcolemma containing Mg^{++} activated ATPase, Na^{+} - K^{+} stimulated, ouabain sensitive ATPase (7 μmoles Pi/mg per h)[59]. The presence of this Mg^{++} independent, Ca^{++} stimulated ATPase in heart sarcolemma has been confirmed[74]. The application of sucrose density gradient technique has been reported to yield plasma membrane containing Mg^{++}ATPase and Na^{+} - K^{+}ATPase (13 μmoles Pi/mg per h)[75]. On the other hand, plasma membranes prepared by exposing the myocardial cell segments to osmotic shock followed by extraction in KCl has been found to contain adenylate cyclase (236 μmoles/mg per min) in addition to Na^{+} - K^{+}ATPase activity (5 μmoles Pi/mg per h)[76]. A critical review of the papers on heart sarcolemma revealed that none of these preparations have been studied and characterized in sufficient detail and the results described thus far appear to be of a preliminary nature. Some reports concerning the isolation and properties of skeletal muscle sarcolemma have also appeared in the literature[19, 77 - 81]. Thus, there is a clear need for a preparation containing adequate activities of Mg^{++}ATPase, Ca^{++}ATPase, Na^{+} - K^{+}ATPase and adenylate cyclase in order to gain further information about cardiac plasma membrane function.

3. Adenylate Cyclase and Na^{+} - K^{+}ATPase of Normal and Failing Hearts

Several hormones and neurotransmitters have been shown to alter adenylate

cyclase activity in the myocardium[13 - 16]. The cytochemical studies have revealed that the adenylate cyclase activity in the cardiac muscle is not only localized at the sarcolemma but is also present in the sarcotubular system within the cell[82]. Association of adenylate cyclase with the sarcoplasmic reticulum has also been demonstrated biochemically[83 - 85]. The properties of heart adenylate cyclase in washed cell particles and in sarcotubular vesicles have been extensively studied by different investigators[85 - 87]. Both catecholamines and NaF have been shown to activate adenylate cyclase by increasing V_{max} without any changes in K_m. The true substrate for this enzyme appears to be MgATP. The integrity of the lipoprotein complex seems to be essential for the full expression of the enzyme activity[85] and phospholipids are necessary components of this enzyme system[88]. Although, as indicated earlier, the presence of adenylate cyclase in the heart plasma membrane has been demonstrated[76], no detailed report to date is available concerning its properties.

Conflicting reports concerning changes in adenylate cyclase activity in heart failure have appeared in the literature. Sobel et al.[89] have reported a reduction in adenylate cyclase activity in failing guinea pig hearts, whereas other investigators have failed to observe such a change[90]. Although Gold et al.[91] did not observe any alteration in adenylate cyclase activity in the absence or presence of norepinephrine or NaF in chronic heart failure induced in cats by occluding the pulmonary artery, glucagon was found unable to activate this enzyme. The isolated right ventricle failure led not only to a decreased capacity of right ventricular adenylate cyclase to respond to glucagon stimulation, but

also to a similar impairment of adenylate cyclase derived from the unstressed left ventricle[92]. A marked reduction in the adenylate cyclase activity in the presence of epinephrine and NaF was observed in hearts which failed to generate contractility due to substrate lack, however, no alteration in the enzyme activity was noted in hearts which failed to generate contractile force by about 50% of the control value[93]. Likewise, no appreciable changes in adenylate cyclase were seen in myopathic hamsters (BIO 14.6) with a moderate degree of heart failure, whereas its responses to norepinephrine and NaF were markedly altered in late stages of failure[28]. The discrepancy in results from various laboratories may be due to either the difference in the type of heart failure or the degree of heart failure.

In spite of a great deal of effort the precise role of adenylate cyclase in myocardial function remains unclear. Although denervation supersensitivity in myocardium has been recognized, the adenylate cyclase activity in the absence and presence of epinephrine in the chronically denervated cat heart was found to be normal[94]. Likewise normal myocardial adenylate cyclase activity has been reported in hyperthyroid cats[95]. On the other hand, Levey et al.[96] have observed a decrease in adenylate cyclase activity in hypothyroidism. An increase in adenylate cyclase activity in washed cell particles obtained from overloaded left ventricles of the perfused guinea pig heart has been observed to be associated with an increased protein synthesis[97]. On the other hand, depressed cardiac adenylate cyclase activity in both hypertrophied and failing hearts due to aortic constriction in rabbits has been demonstrated[98]. Such conflicting results point out

the need for further studies on adenylate cyclase with respect to changes in myocardial function. To the best of our knowledge, no report exists in the literature concerning changes in sarcolemmal adenylate cyclase under conditions of depressed cardiac performance.

As in the case of adenylate cyclase, cytochemical evidence[99, 100] has also accumulated to support the localization of $Na^+ - K^+$ATPase in the sarcolemma following the demonstration of this enzyme in cardiac tissue[101 - 107]. The properties of $Na^+ - K^+$ATPase from various tissues have been extensively studied[17, 18, 108 - 112]. This enzyme requires both Na^+ and K^+ for full activation and uses MgATP as substrate. Both ouabain and Ca^{++} are well known inhibitors of this enzyme. The kinetics as well as cardiac glycoside binding data suggest this is an allosteric enzyme possessing multiple ligand binding sites[18, 113, 114]. There is also a sufficient body of evidence to indicate a specific inhibitory effect by digitalis glycosides on the $Na^+ - K^+$ATPase activity of hearts from various species[115 - 122]. The well known differences in the sensitivity of various species to cardiac glycosides has been explained on the basis of relative affinity of $Na^+ - K^+$ATPase to the drug[116, 117]. It should be pointed out that most of the above work with $Na^+ - K^+$ATPase has been carried out with a solubilized enzyme preparation and little is known concerning the properties of the enzyme in its native state in the cardiac sarcolemma.

In addition to the suggested role of $Na^+ - K^+$ATPase in the transport of Na^+ and K^+ across the cell membrane this enzyme has been considered to have an important function in the transport of sugars, amino acids and biogenic amines[18].

The positive inotropic effect of ouabain has been shown to occur concomitantly with an inhibitory effect on $Na^+ - K^+$ ATPase[123, 124]. Studies by Prasad[125] have demonstrated a relation between the inhibition of $Na^+ - K^+$ ATPase and shortening of action potential duration. Recently, increased Ca^{++} entry during the plateau of the action potential has been proposed to occur as a possible mechanism of cardiac glycoside action on $Na^+ - K^+$ ATPase[126]. All of these studies reflect the importance of $Na^+ - K^+$ ATPase in a wide variety of physiological and pharmacological events in the myocardium but do not clearly elucidate the exact mechanism of its involvement.

Some reports indicating alterations in $Na^+ - K^+$ ATPase activity in different models of failing hearts have appeared in the literature. For example, an increase in $Na^+ - K^+$ ATPase activity has been observed in experimental cardiac insufficiency induced by Co^{++} or vitamin E deficiency[127, 128]. A marked elevation of the enzyme activity was also observed in failing hearts of genetically myopathic hamsters (BIO 14.6)[29]. Similarly cardiac $Na^+ - K^+$ ATPase activity has been found to increase in K^+ deficiency[129]. On the other hand, a decrease in $Na^+ - K^+$ ATPase activity has been reported in failing hearts due to aortic constriction, hypoxia or substrate lack[130 - 132]. Reduction in cardiac $Na^+ - K^+$ ATPase activity has also been observed in animals with hypoadrenalism[133]. Furthermore, low $Na^+ - K^+$ ATPase activity was reported in failing human hearts and these preparations were also less sensitive to inhibition by ouabain[27]. It should be noted that Mead et al. did not find any change in $Na^+ - K^+$ ATPase in failing dog hearts due to pulmonary artery stenosis[134]. These apparently

contradictory results on the $Na^+ - K^+$ ATPase activity in failing hearts could be due to differences in the degree and the type of heart failure. Clearly, more experiments are needed in this area to provide further information.

METHODS

1. Animals

Mongrel dogs (10 - 15 kg) were anesthesized with 30 mg/kg sodium pentobarbitol (intravenously). The hearts from rats (about 300 g) and hamsters were removed following decapitation. Cardiomyopathic hamsters (UM-X7.1) around 200 days old (about 150 g) with severe degree of heart failure were employed. This new line of hamsters has been developed with 100% incidence of congestive heart failure[30]. Normal Syrian hamsters of the same age group were used as controls. All the hearts were rapidly removed and placed in ice cold Tris-HCl buffer, pH 7.4.

2. Chemicals

All the chemicals employed in this study were of analytical grade. The ^{14}C-8-ATP (specific activity 49.3 mcuries/mM) was purchased from New England Nuclear. Deionized distilled water was used throughout this study.

3. Preparation of Membrane Fraction

The dog heart left ventricles (4 g) were washed thoroughly, diced with a pair of scissors, and homogenized with 10 vol. of 10 mM Tris-HCl, pH 7.4, containing 1 mM ethylenediamine-tetraacetate disodium (EDTA) in a Waring Blendor for 1 min (30 s X 2, with an interval of 1 min). The homogenate was filtered through gauze and centrifuged at 1,000 X g for 10 min. The sediment was suspended in 100 ml of the above Tris-buffer and stirred in the cold room for 30 min and centrifuged at 1,000 X g for 10 min. The residue was then suspended

in 100 ml of Tris-buffer, pH 8.0, stirred for 30 min and centrifuged at 1,000 X g for 10 min. The sediment was suspended in 10 mM Tris-HCl, pH 7.4, stirred for 30 min and centrifuged at 1,000 X g for 10 min; this step was then repeated again. The sediment was suspended in 100 ml of 10 mM Tris-HCl, pH 7.4, extracted with 0.4 M LiBr for 45 min and centrifuged at 1,000 X g for 10 min. This sediment was then washed with 10 mM Tris-HCl, pH 7.4, and suspended in 1 mM Tris, pH 7.4, and immediately used. All the above steps were performed at 0 - 4°C. In addition to isolating these membranes from the dog heart, we have also applied this method to rat and hamster hearts. The fraction isolated in the above described manner will be referred to as "membrane fraction" or "membranous fraction". The isolation procedure outlined above took about 5 h. This method is a modification of that described by Kono and Colowick[135] and is essentially similar to that described by Severson et al.[80] for the skeletal muscle membranes.

In some experiments, the 0.4 M LiBr extracted and washed fraction was further extracted for 45 min with 2 M NaI, washed and immediately used; this fraction will be referred to as "NaI treated membrane fraction".

4. Preparation of Solubilized $Na^+ - K^+$ ATPase Enzyme

The hearts were placed in ice-cold medium (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.2 - 7.4), washed thoroughly to remove blood and homogenized with 15 volumes of the above medium for 40 s (20 s X 2) in a Waring Blendor in a cold room (0 - 4°C). The homogenate was filtered through

several layers of gauze, centrifuged at 10,000 X g for 15 min, the residue homogenized (original volume) for 20 s with the above medium containing 0.1% deoxycholate, stirred for 20 min and spun at 10,000 X g for 10 min. The sediment was discarded and the clear supernatant, after filtration, was centrifuged at 70,000 X g for 1 h. The residue was suspended in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.2 - 7.4 containing 0.05% deoxycholate with the aid of a glass Teflon homogenizer, stirred at 0 - 4°C for 15 - 20 min and then centrifuged at 10,000 X g for 10 min. The residue was discarded and the supernatant after filtration, was centrifuged at 70,000 X g for 1 h. The residue thus obtained was thoroughly washed and suspended in 1 mM Tris-EDTA solution (pH 7.0) and centrifuged at 70,000 X g for 1 h. This process was repeated twice, the sediment suspended in 1 mM Tris-EDTA. This preparation was treated further as follows: the suspension was stirred slowly in an ice bath with 2 M sodium iodide, 5 - 10 mM $MgCl_2$, 15 mM EDTA, 100 mM Tris-HCl, pH 8.0 for 45 min, diluted 2.5 times with 1 mM Tris-EDTA, and centrifuged at 38,000 X g for 45 min. The final residue was obtained by a repeated cycle of washing, resuspending and centrifuging in 1 mM Tris-HCl and used immediately. The principle of this procedure is based on the method described by Matsui and Schwartz[108].

5. Determination of Enzyme Activities

About 50 - 100 μg of the membrane protein was employed for each reaction in this study. Each enzyme reaction was linear with respect to the time and protein concentration. Fresh membrane preparations were used for each experiment. All the results were analyzed statistically according to the

conventional student "t" test.

a) $Na^+ - K^+$ ATPase

For routine estimation of the $Na^+ - K^+$ stimulated ATPase activity, the fractions were incubated in a total volume of 1 ml containing 50 mM Tris-HCl, pH 7.4 - 7.6, 4 mM $MgCl_2$, 100 mM NaCl and 10 mM KCl in the presence or absence of ouabain (2 mM). After 5 min of pre-incubation at 37°C, the reaction was started by addition of ATP (4 mM final concentration) and the fraction was further incubated for 10 min. The reaction was stopped by the addition of 1 ml of 12% cold trichloroacetic acid, centrifuged and the Pi in the clear supernatants was determined. The difference of the activities in the absence and presence of ouabain is referred to as $Na^+ - K^+$ stimulated, Mg^{++} dependent, ouabain sensitive ATPase ($Na^+ - K^+$ ATPase) and the values thus obtained agreed very well with the ATP hydrolysis due to the presence of Na^+ and K^+ in the incubation medium.

b) Mg^{++} ATPase

The Mg^{++} ATPase activity was assayed in a total volume of 1 ml containing 50 mM Tris-HCl, pH 7.4 - 7.6, 4 mM Mg^{++}, and 4 mM Tris-ATP. After 5 min of pre-incubation at 37°C of the enzyme preparation in the above medium, the reaction was started by addition of ATP and the fraction was further incubated for 10 min. The reaction was stopped by the addition of 1 ml of 12% cold trichloroacetic acid, the tubes centrifuged and the Pi in the clear supernatants was determined. The ATP hydrolysis that occurred in the absence of Mg^{++} was subtracted in order to calculate the activity due to Mg^{++} stimulated ATPase.

c) Ca^{++} ATPase

The Ca^{++} ATPase activity was assayed in a total vol. of 1 ml containing 50 mM Tris-HCl, pH 7.4 - 7.6, 4 or 8 mM Ca^{++} and 4 mM Tris-ATP. After 5 min of pre-incubation at 37°C of the enzyme preparation in the above medium, the reaction was started by addition of ATP and the fraction was further incubated for 10 min. The reaction was stopped by the addition of 1 ml of 12% cold trichloroacetic acid, the tubes centrifuged and the Pi in the clear supernatants was determined. The ATP hydrolysis that occurred in the absence of Ca^{++} was substracted in order to calculate the activity due to Ca^{++} stimulated ATPase.

d) Adenylate Cyclase

The adenylate cyclase activity was assayed at 37°C in a total vol. of 0.15 ml containing 50 mM Tris-HCl, pH 8.5, 8 mM caffeine, 5 mM KCl, 20 mM phosphoenol pyruvate, 15 mM $MgCl_2$, 130 µg/ml pyruvate kinase and 0.4 mM ATP-^{14}C unless otherwise stated. The reaction was started by the addition of ATP, and stopped by boiling the tubes for 3 min under conditions preventing evaporation, with prior addition of cold cyclic AMP (final concentration 1.5 - 2.0 mM). The tubes were centrifuged and 100 µl of the clear supernatant spotted on Whatman No. 3 MM paper for descending chromatography, using 1 M ammonium acetate : 95% ethanol (15 : 35). After drying, the cyclic AMP spot was visualized by ultra-violet light. The area containing cyclic AMP was cut and counted in 20 ml of Bray's solution in a Packard liquid scintillation counter. The non-enzymatic formation of cyclic AMP was estimated by denaturing the enzyme preparation by

boiling prior to the addition of ATP. The above method is essentially similar to that described by Drummond and Duncan[86].

6. Electron Microscopy

The membrane fraction of the dog heart was prepared by hypotonic shock and LiBr treatments as described above. The pellets were fixed overnight in 5% gluteraldehyde in a 0.1 M phosphate buffer, pH 7.4, diced into pieces of about 0.5 mm thickness, washed overnight in 0.1 M phosphate buffer, pH 7.4, post fixed for 1 h in 1% osmium tetraoxide. The membranous fraction pellet was dehydrated in a graded ethanol series (50 - 100%) and embedded in Araldite-502 epoxy resin. Thin sections were made on a Porter-Blum MTII ultramicrotome using glass knives and stained with Reynold's lead citrate. The sections were examined and photographed using a Zeiss BM93 electron microscope.

7. Marker Enzyme and Calcium Binding Activities

The mitochondria and heavy microsomes were isolated from the myocardium by the method described by Sulakhe and Dhalla[12]. The calcium binding was determined by Millipore filtration technique as described by Sulakhe and Dhalla[12]. The activities of the marker enzymes, glucose-6-phosphatase and cytochrome C oxidase, were determined according to the methods described elsewhere[12, 136, 137]. Acid phosphatase was estimated by determining Pi released[138] following the incubation of the membrane fraction with 25 mM β-glycerol phosphate buffered at pH 5.0 with 50 mM acetate in a final vol. of 200 µl for 20 min at 37°C.

8. Determination of Inorganic Phosphate (Pi) and Protein

The estimation of Pi was carried out by the method of Taussky and Shorr[138]. Usually 0.5 ml or 1.0 ml of the clear supernatant was employed for the estimation of phosphate. Each time a standard curve for Pi was constructed to ensure the reliability of phosphate values. Not more than 15% of the added ATP was hydrolyzed under the present experimental conditions. This eliminated the necessity for the use of ATP regenerating system during the assay of the ATPase activity. Protein concentration was estimated by the method of Lowry et al.[139].

9. Rat Heart Perfusion

Each male rat weighing about 300 g was sacrificed by decapitation, the heart rapidly removed, placed in ice cold oxygenated Krebs-Henseleit bicarbonate buffer and freed from adipose and connective tissue. The aorta was tied to a cannula of the perfusion apparatus for coronary perfusion by the conventional Langendorff technique as described elsewhere[140, 141]. After equilibrating the hearts for 10 min with control perfusion medium, the hearts were perfused with a medium of desired ionic composition for varying time periods as indicated in the text. The coronary flow was maintained at a rate of 10 ml/min with a peristaltic pump. Krebs-Henseleit bicarbonate buffer of the following composition (mM) was used as a control medium in all experiments: Na^{+}, 145; K^{+}, 6; Mg^{++}, 1.2; Ca^{++}, 1.25; Cl^{-}, 126; HCO_3^{-}, 25; $SO_4^{=}$, 1.2; $PO_4^{\equiv}$, 1.2 and dextrose, 11.1. When any cation was omitted in the medium, the osmolarity

was maintained by adding an appropriate amount of sucrose. The perfusion medium was equilibrated with 95% O_2 and 5% CO_2 gas mixture and the pH of the medium was 7.4. The temperature of the perfusion medium was maintained at 37°C. The force of contraction was measured using a strain gauge force transducer (FT.03)[141].

RESULTS

1. Characteristics of Membrane Fraction

The membrane fraction isolated from the dog heart was found to contain ouabain sensitive, $Na^+ - K^+$ stimulated ATPase (about 10 μmoles Pi/mg per h). It can be seen from the data in Table I that approximately 90% of the total $Na^+ - K^+$ stimulated ATPase in the heart homogenate was present in the membrane fraction. The protein yield of the dog heart membranes varied from 20 - 22 mg/g. The fraction also contained Mg^{++}ATPase (about 16 μmoles Pi/mg per h) (Table II). The $Na^+ - K^+$ATPase activity in this fraction was 7 - 8 fold, whereas Mg^{++}ATPase was 2 fold as compared to the homogenate.

The electron microscopic examination of the dog membrane fraction did not reveal the presence of intact mitochondria or myofibrils. This fraction appears to consist of empty membranous sacs of varying shapes and sizes (Fig. 1). The absence of ATP supported Ca^{++} binding by the dog heart membrane fraction also supports the absence of contaminating mitochondria and sarcotubular vesicles (Table III). The results shown in Table IV indicate that both mitochondrial and microsomal fractions did not contain any demonstrable ouabain sensitive, $Na^+ - K^+$ stimulated ATPase. The specific activities of cytochrome C oxidase, glucose-6-phosphatase and acid phosphatase were very low in the membranous fraction in comparison with the mitochondrial and sarcoplasmic reticular fractions (Table IV). It can also be seen in Table IV that the membranous fraction contained high adenylate cyclase activity. The specific activity of the adenylate cyclase in the dog heart membrane fraction was 7 - 8 fold of that in the heart homogenate.

TABLE I

THE YIELD OF PROTEIN AND THE RECOVERY OF OUABAIN SENSITIVE $Na^+ - K^+$ STIMULATED ATPase FROM DOG HEART VENTRICLES

The results are obtained from 3 g heart tissue in a typical experiment with 4 such preparations. The specific activity is expressed as ouabain sensitive μmoles Pi released/mg protein per h at 37°C. The assay system was the same as in Methods.

Fraction	Total protein (mg)	Percent protein	Total activity	Percent activity	Specific activity
Homogenate	410	100.0	738	100.0	1.8
1,000 X g sediment (washed X 2)	220	53.6	572	77.5	2.6
Membranous fraction	65	15.9	659	88.2	10.1

TABLE II

ATPase ACTIVITIES OF VARIOUS FRACTIONS ISOLATED FROM DOG HEART HOMOGENATE

The activities of Mg^{++}ATPase and Na^{+}- K^{+} stimulated ATPase are expressed as μmoles Pi/mg protein per h at 37°C. The assay systems were the same as in Methods. The values represent typical results of duplicate determinations on 3 separate preparations.

Fraction	Mg^{++} ATPase	Na^{+}- K^{+} ATPase	$\frac{Na^{+}\text{- }K^{+}\text{ ATPase}}{Mg^{++}\text{ ATPase}}$	Fold purification of Na^{+}- K^{+} ATPase
Homogenate	8.1	1.3	0.18	1.0
1,000 X g sediment (washed X 2)	8.1	1.7	0.21	1.3
Membranous fraction	16.5	10.1	0.61	7.7

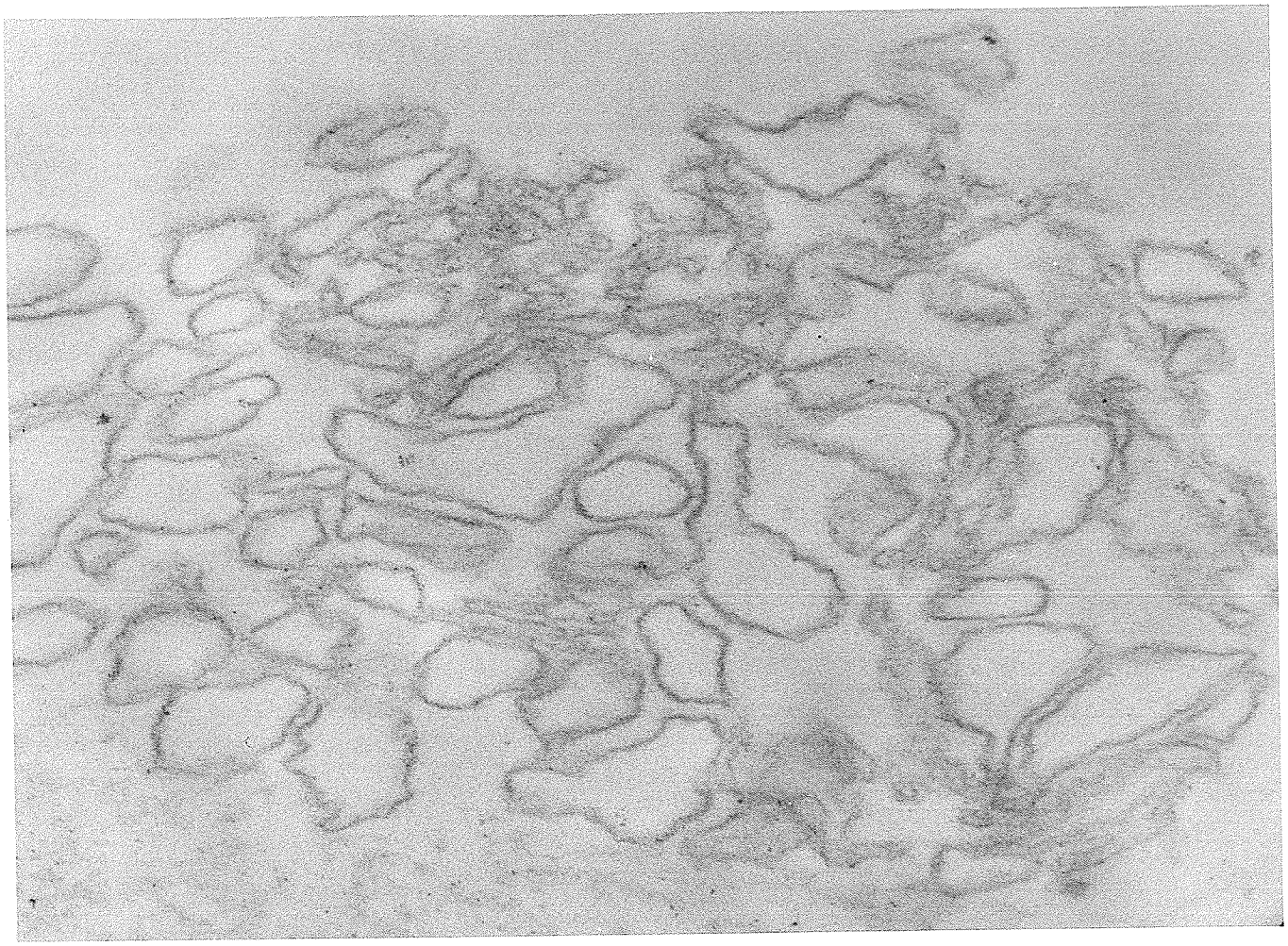

FIGURE 1 Electron micrograph of typical membrane preparation isolated from dog heart left ventricle as described in Methods. (X 29,300).

TABLE III

CALCIUM BINDING BY DOG HEART SUBCELLULAR FRACTIONS IN THE ABSENCE OR PRESENCE OF ATP

Each fraction (0.3 - 0.4 mg protein/ml) was incubated in medium containing 100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl (pH 6.8) and 0.1 mM $^{45}CaCl$ at 37°C. Calcium binding was determined as described in Methods. Each value is a mean ± S.E. of 4 experiments.

Fraction	Calcium binding (nmoles/mg protein per 5 min)	
	Absence of ATP	Presence of 2 mM ATP
Mitochondria	10 ± 1.5	32 ± 2.6
Microsomes	8 ± 1.4	42 ± 3.1
Membranes	14 ± 2.0	15 ± 1.8

TABLE IV

MARKER ENZYME ACTIVITIES OF DIFFERENT FRACTIONS ISOLATED FROM DOG HEART HOMOGENATE

Cytochrome C oxidase activity is expressed as nmoles cytochrome C oxidized/mg protein per min at 27°C, whereas the activities of Na^+- K^+ stimulated ATPase, glucose-6-phosphatase and acid phosphatase are expressed as µmoles Pi/mg protein per h at 37°C. The adenylate cyclase activity is expressed as pmoles cyclic AMP/mg protein per min at 37°C. The assay systems were the same as in Methods. The values represent typical results of duplicate determinations on 4 separate preparations.

Fraction	Na^+- K^+ ATPase	Adenylate cyclase	Cytochrome C oxidase	Glucose-6-phosphatase	Acid phosphatase
Membrane	10.50	285	25	0.18	0.06
Mitochondria	0.03	56	1,200	0.12	0.22
Microsome	0.05	70	75	1.25	0.10

In addition, Mg^{++} independent, Ca^{++}ATPase (about 15 μmoles Pi/mg per h) which is considered to be mainly localized at the cell membrane[19, 59] was also present in this fraction.

In order to further establish the possible contamination by cytoplasmic components, different fractions such as mitochondria, microsomes and 40,000 X g supernatant were added to the heart homogenate and the membranous fraction was isolated. As can be seen in Table V the specific activities of Mg^{++}ATPase, Na^{+} - K^{+}ATPase, Ca^{++}ATPase and adenylate cyclase of fractions obtained from heart homogenates, in the presence of various subcellular additions were not different from the controls. Although we consider this sarcolemmal fraction to be relatively free from major cytoplasmic contaminants, it should be recognized that some inert non-sarcolemmal proteins are present in this fraction. This is based upon our experiments using discontinuous sucrose density gradients which resulted in a 10 - 15% increase in the specific activity of Na^{+} - K^{+}ATPase. We do not consider sucrose density gradient purification to be desirable in view of the additional 3 - 4 h required to achieve only a slight increment in the enzyme activity.

2. Na^{+} - K^{+}ATPase Activity of Membrane Fraction

The Na^{+} - K^{+}ATPase activity of the dog heart membrane fraction was studied at different concentrations of MgATP and the results are shown in Fig. 2. The Lineweaver-Burk[142] plot analysis of the data revealed that the values for V_{max} and K_m were 13 ± 1.2 μmoles Pi/mg per h and 0.77 ± 0.1 mM respectively. On treating the membrane fraction with 2 M NaI or 2 M LiBr the specific activity

TABLE V

ENZYME ACTIVITIES OF MEMBRANE FRACTIONS OBTAINED FROM DOG HEART HOMOGENATE IN THE ABSENCE OR PRESENCE OF DIFFERENT SUBCELLULAR FRACTIONS

Mitochondrial, microsomal and 40,000 X g supernatant fractions were obtained from 5 g of heart tissue. Each of these fractions was added to the homogenates (5 g heart tissue in each case) and the membranous fractions were isolated according to the method described in the text. Na^{+}- K^{+}ATPase, Mg^{++}ATPase, Ca^{++}ATPase activities are expressed as μmoles Pi/mg protein per h, whereas adenylate cyclase activity is expressed as pmoles cyclic AMP/mg protein per min. The assay systems were the same as in Methods. The results are typical of 3 such experiments.

Additions in the homogenate before isolating the membranous fraction	Mg^{++} ATPase	Na^{+}- K^{+} ATPase	Ca^{++} ATPase	Adenylate cyclase
-	16.3	9.8	14.5	270
Mitochondria	16.2	10.1	14.0	281
Microsomes	15.9	10.0	14.3	275
40,000 X g supernatant	16.3	10.3	14.4	268

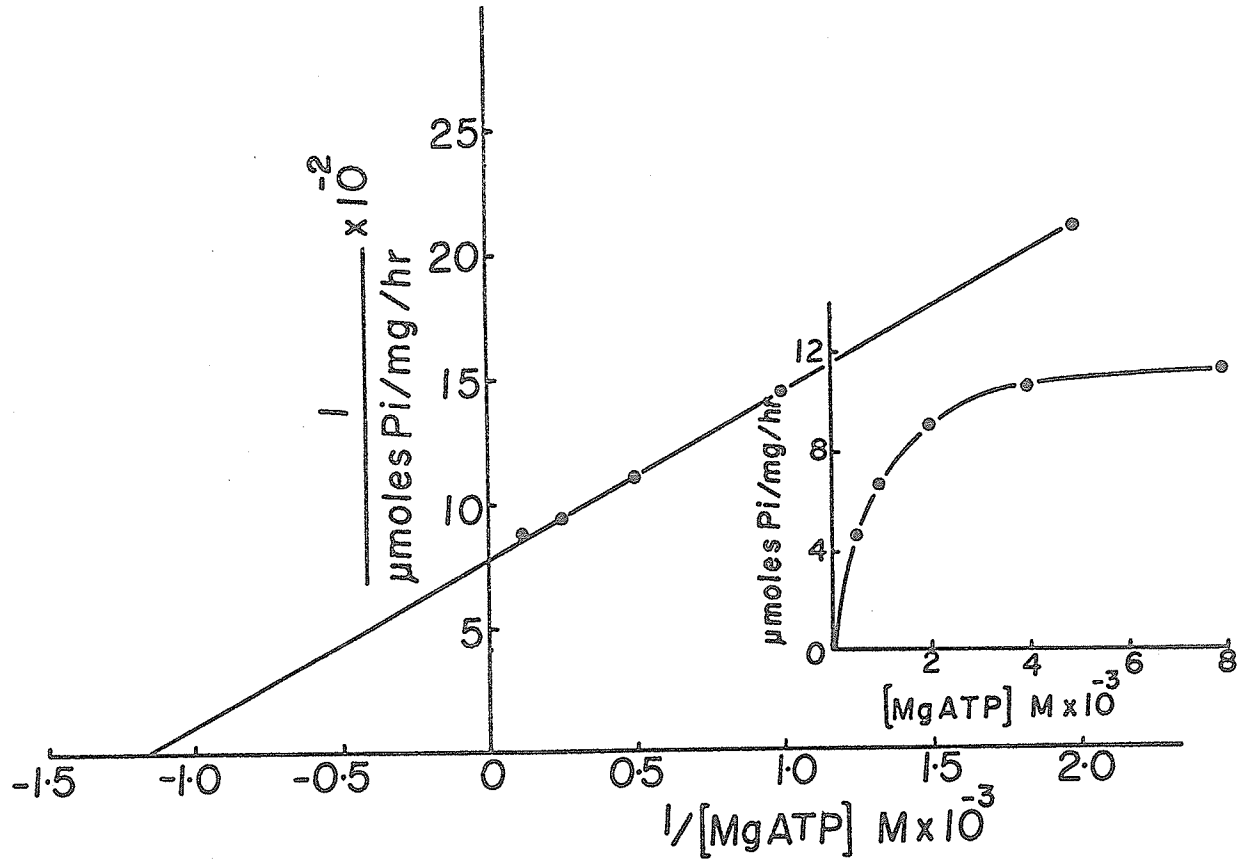

FIGURE 2 Effect of MgATP on the dog heart membrane Na^+-K^+ATPase activity. The assay system was the same as in Methods except that the concentration of MgATP was varied as indicated. The results are typical of 4 experiments.

of $Na^+ - K^+$ ATPase was increased by about 50% without appreciable changes in Mg^{++} ATPase or Ca^{++} ATPase (Table VI); however, this high salt treatment markedly reduced the activity of adenylate cyclase in the membranous fraction. When the $Na^+ - K^+$ ATPase activities of these dog heart membrane fractions were compared with the solubilized enzyme preparations, the enzyme activities of the membrane fraction, with or without NaI treatment, were 20 - 25% of that found in the solubilized preparation (Table VII). However, the enzyme activities in these fractions obtained in hearts from rat and hamster were 50 - 66% and 33 - 50% of the solubilized preparation respectively. The pH optimum for $Na^+ - K^+$ ATPase activity of the NaI treated membrane fraction was found to be 7.5; the results are shown in Fig. 3. When the ratio of Na^+/K^+ in the incubation medium was held constant, the maximal $Na^+ - K^+$ ATPase activity was obtained at 20 mM Na^+/2 mM K^+ (Fig. 4) in the NaI treated membrane fraction. Monovalent ions, such as Rb^+, NH_4^+, Cs^+, Li^+ and choline were poor substitutes for K^+ in activating the enzyme present in the NaI treated membrane fraction (Fig. 5). Results similar to those found with NaI treated membrane fractions were also obtained with the membranous fraction and the solubilized enzyme preparation.

In another set of experiments, the concentrations of MgATP, K^+ or Na^+ were varied in the incubation medium and the $Na^+ - K^+$ ATPase activity of the solubilized enzyme preparation was studied and the results are shown in Figs 6 - 8 respectively. In all three cases the activity curves were sigmoidal and the n values, obtained by Hill plots[143], were greater than one. Both Ca^{++}

TABLE VI

EFFECT OF NaI OR LiBr TREATMENT ON THE ENZYME ACTIVITY OF THE DOG HEART MEMBRANE FRACTION

The membrane fraction obtained as described in Methods was further treated with either NaI or LiBr for 45 min, washed and the enzyme activities assayed as in Methods. Results shown for Mg^{++}ATPase, Na^{+}- K^{+}ATPase and Ca^{++}ATPase are expressed as μmoles Pi/mg protein per h; those for adenylate cyclase are expressed as pmoles cyclic AMP formed/mg protein per min. Each value is a mean ± S.E. of 5 experiments.

Treatment	Mg^{++}ATPase	Na^{+}- K^{+} ATPase	Ca^{++}ATPase	Adenylate cyclase
-	15.9 ± 1.2	9.8 ± 0.4	15.0 ± 0.9	297 ± 26
NaI (2 M)	12.9 ± 1.7	15.6 ± 0.7	12.2 ± 1.9	56 ± 6
LiBr (2 M)	13.5 ± 1.4	13.9 ± 0.6	14.0 ± 0.8	127 ± 21

TABLE VII

Na^+- K^+ATPase ACTIVITIES OF DOG, RAT AND HAMSTER HEART PREPARATIONS OBTAINED BY DIFFERENT PROCEDURES

Results are shown as μmoles Pi/mg protein per h. The various fractions were obtained and assayed as outlined in the Methods. Each value is a mean ± S.E. of 6 experiments.

Procedures	Dog	Rat	Hamster
Membrane fraction	10.0 ± 0.8	12.5 ± 1.1	7.8 ± 0.7
NaI treated membrane fraction	16.2 ± 1.4	14.1 ± 1.7	11.5 ± 0.9
Solubilized enzyme preparations	48.4 ± 2.1	21.5 ± 1.2	23.2 ± 1.4

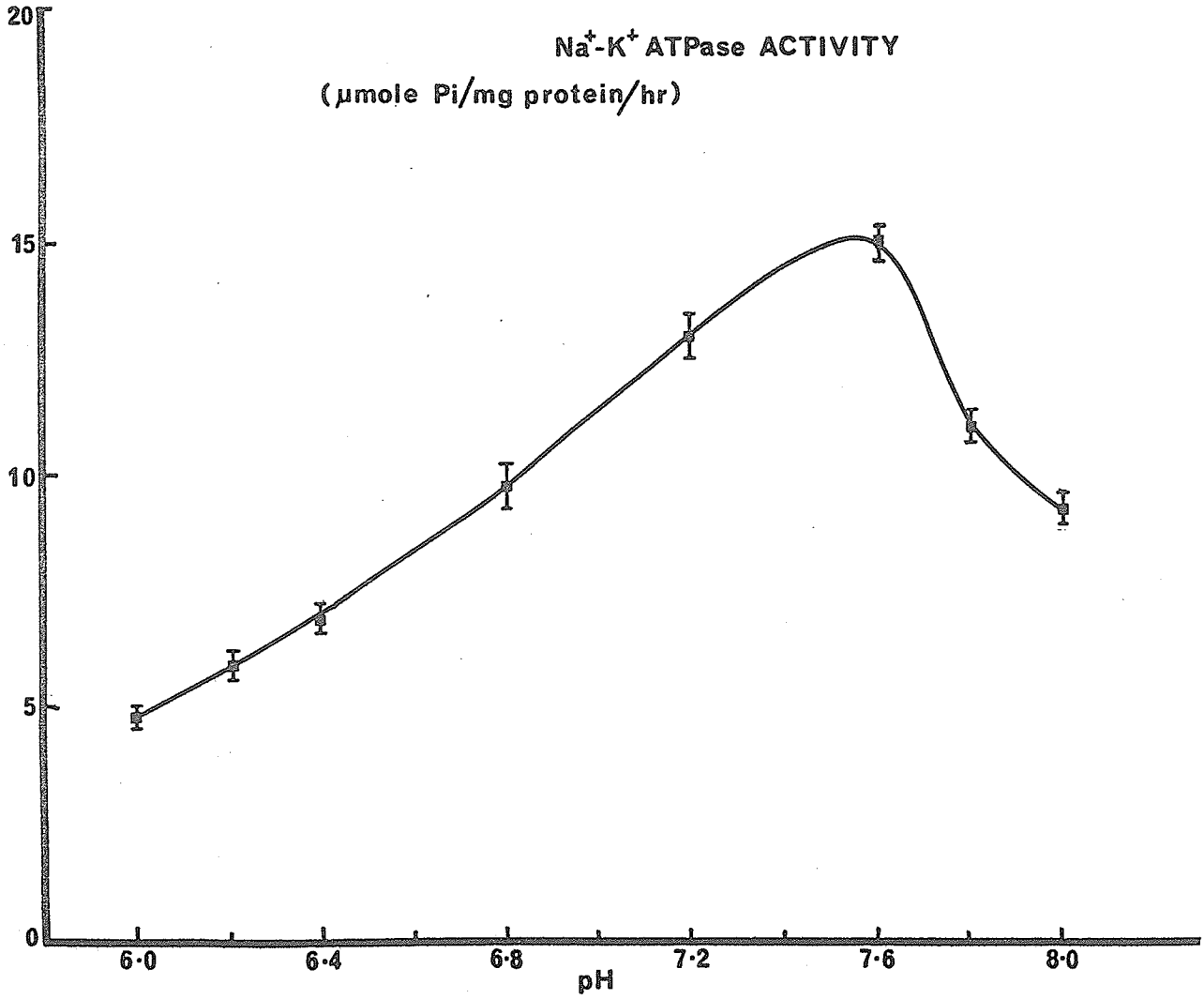

FIGURE 3 Effect of pH on the $Na^+ - K^+$ ATPase activity of the dog heart NaI treated membrane fraction. The assay system was the same as in Methods except that the pH of the incubation medium was varied as indicated. Each value is a mean ± S.E. of 4 experiments.

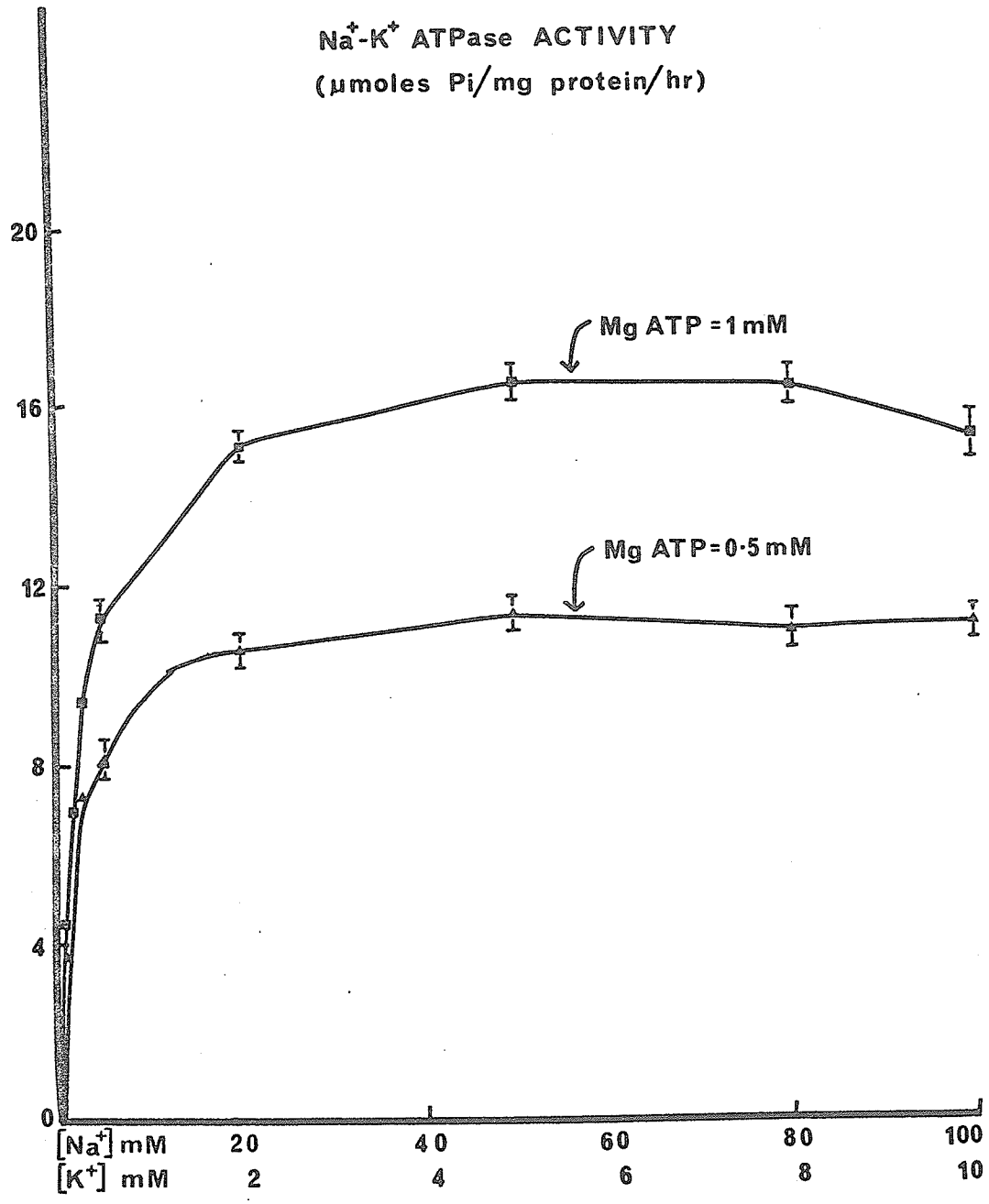

FIGURE 4 Effect of varying the Na^+ and K^+ concentration keeping the Na^+/K^+ constant on the Na^+- K^+ATPase activity of the dog heart NaI treated membrane fraction. The assay system was the same as in Methods except as indicated. Each value is a mean ± S.E. of 3 experiments.

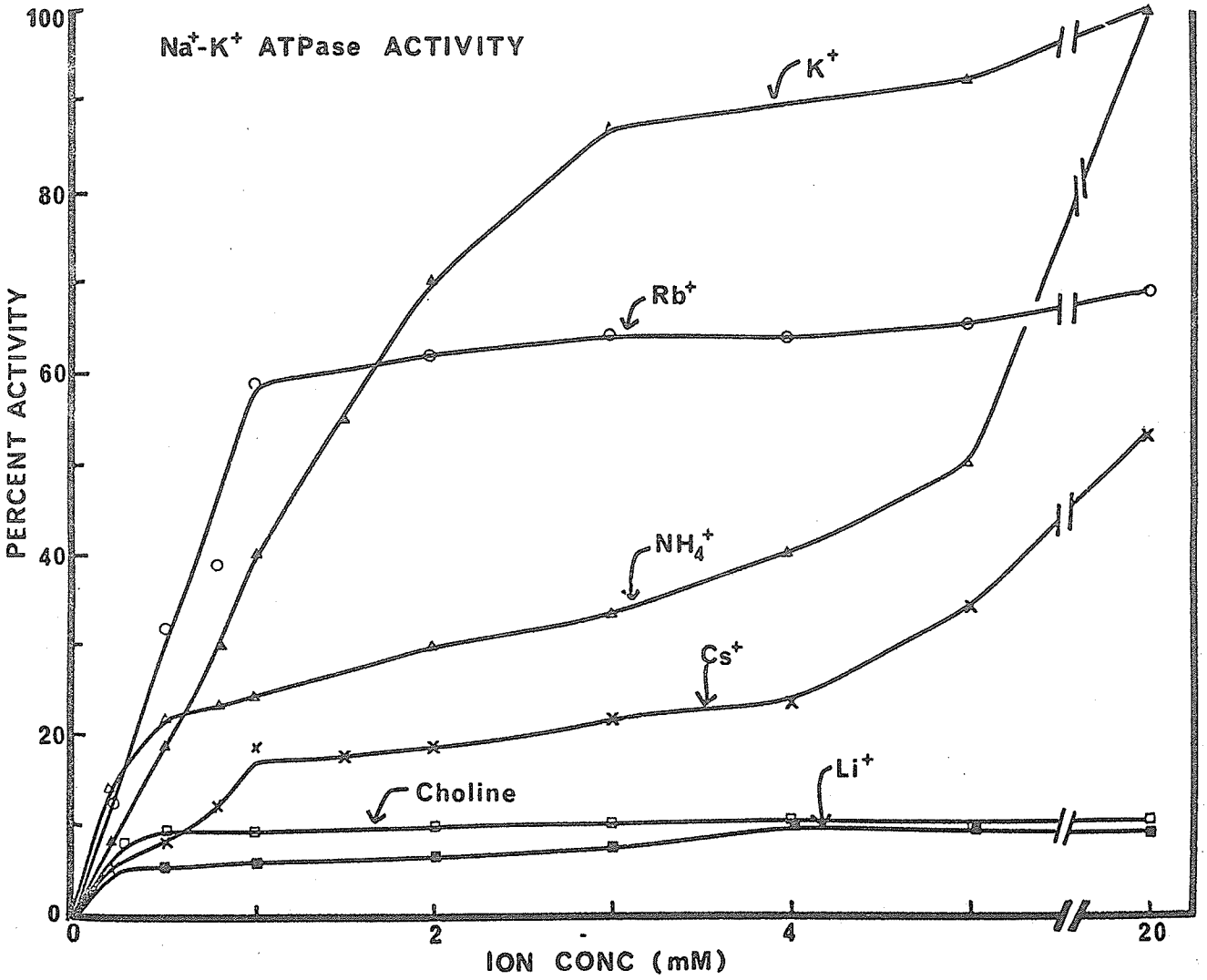

FIGURE 5 Effect of substitution of other monovalent cations for K^+ on the Na^+- K^+ATPase activity of the dog heart NaI treated membrane preparation. The assay system was the same as in Methods except for K^+ substitution. Results are typical of 3 preparations.

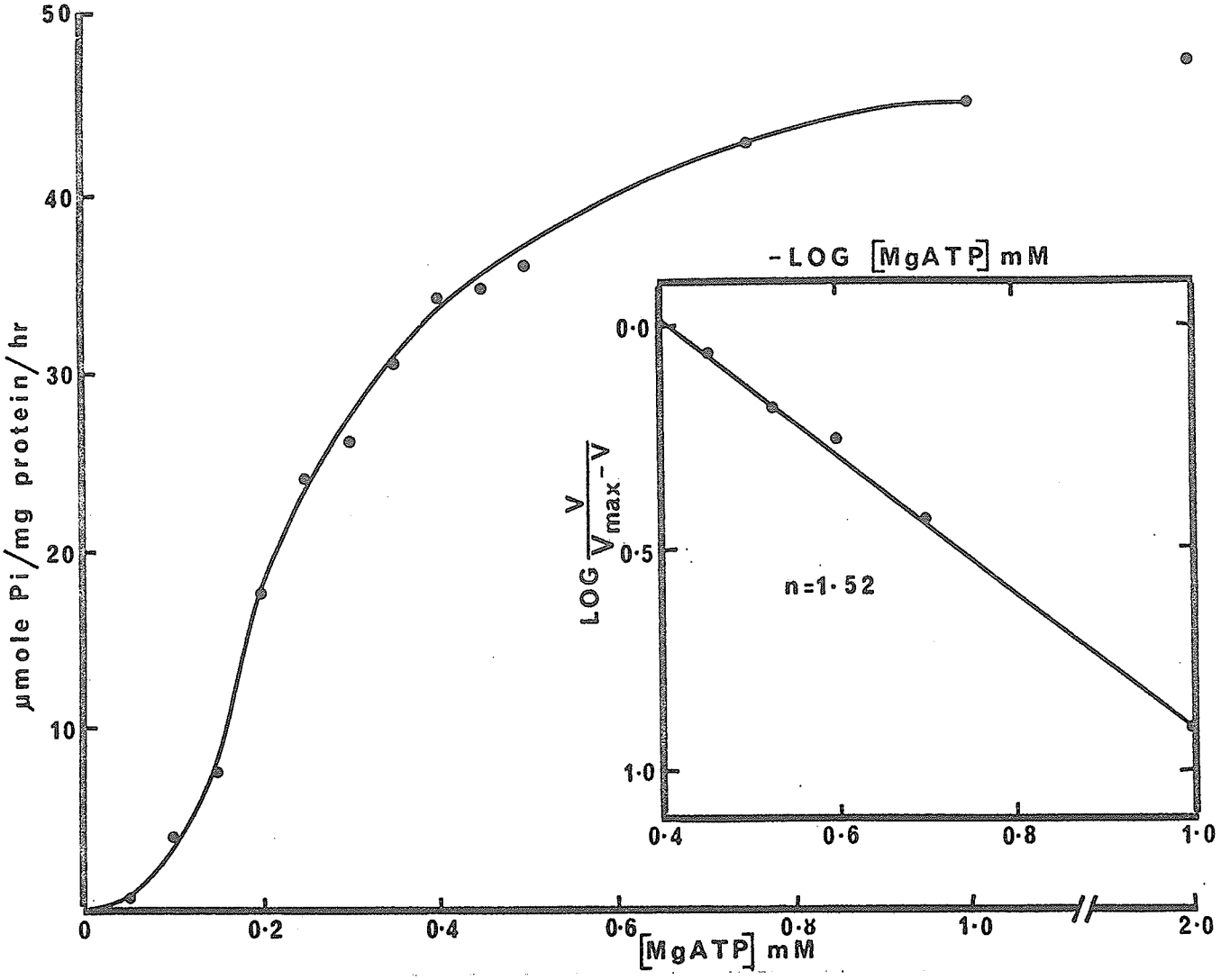

FIGURE 6 Effect of varying MgATP concentration ($[Mg^{++}]$)/ $[ATP]$ = 1/1) on the Na^{+}-K^{+}ATPase activity of the dog heart solubilized enzyme preparation. Insert is a Hill plot of this data. Conditions of incubation were the same as in Methods except for varying MgATP concentrations. Results are typical of 4 such preparations.

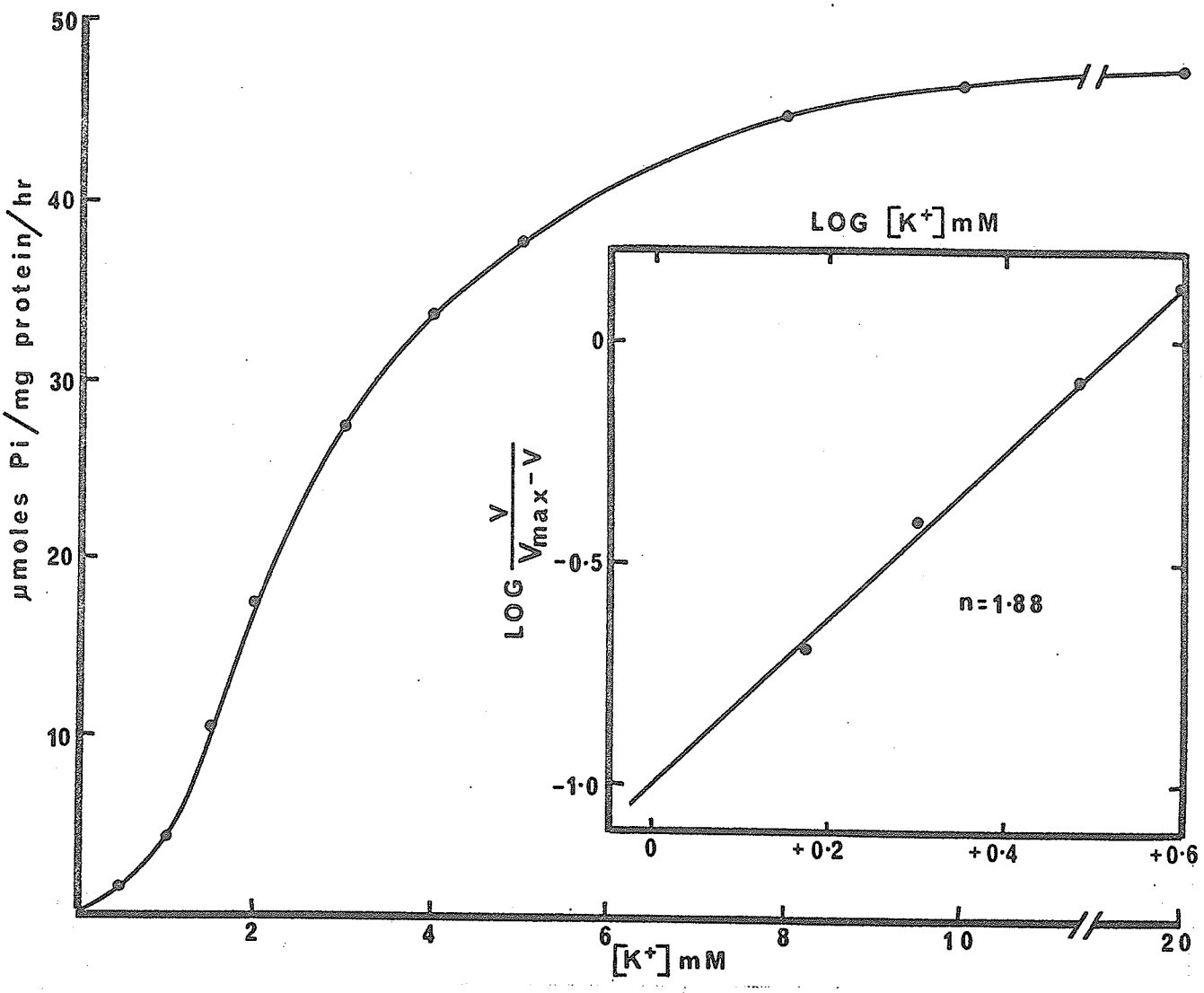

FIGURE 7 Effect of varying K^+ concentrations on the $Na^+ - K^+$ ATPase activity of the dog heart solubilized enzyme preparation. Insert is a Hill plot of the data. Conditions of incubation were the same as in Methods except for varying K^+ concentrations. Results are typical of 4 such preparations.

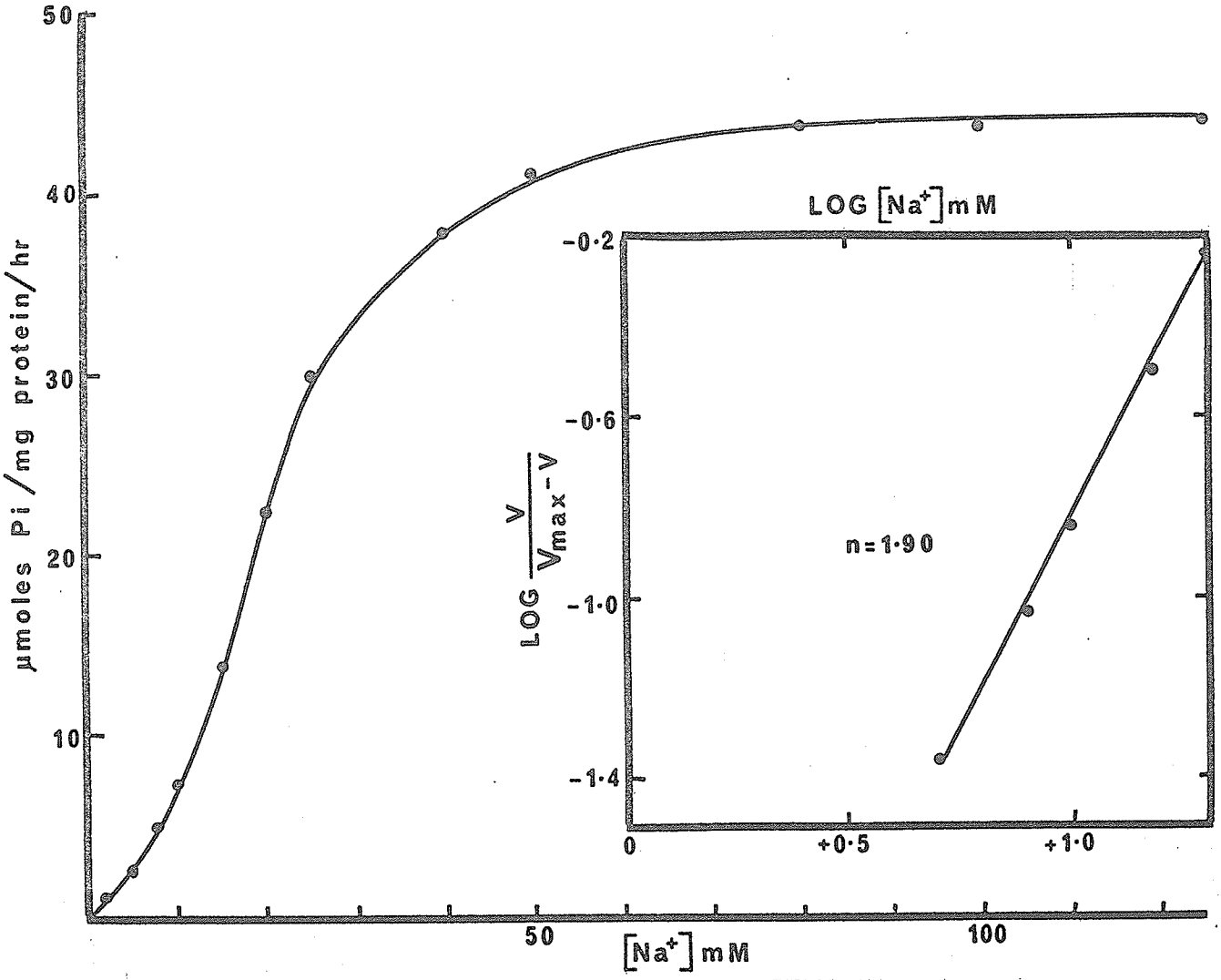

FIGURE 8 Effect of varying Na^+ concentrations on the $Na^+ - K^+$ ATPase activity of the dog heart solubilized enzyme preparation. Insert is a Hill plot of the data. Conditions of incubation were the same as in Methods except for varying Na^+ concentrations. Results are typical of 4 such preparations.

and ouabain were found to markedly inhibit the $Na^+ - K^+$ ATPase activity of the dog heart solubilized enzyme preparation (Fig. 9); however, the inhibitory effect of these agents was found to be pH dependent (Fig. 10).

Table VIII contains the data concerning the comparison of some properties of the membranous fractions with the solubilized enzyme preparation. The characteristics of $Na^+ - K^+$ ATPase described in this table are essentially similar in all three fractions except for the K_m values for the solubilized preparation which were markedly lower than those for the membranous fractions. Various sympathomimetic agents and cyclic AMP failed to alter the ATPase activities of these three preparations (Table IX).

3. Ca^{++} ATPase, Mg^{++} ATPase and Adenylate Cyclase Activities of Membrane Fraction

The dog heart membranes were found to contain a considerable amount of ATP hydrolyzing activity due to the presence of Ca^{++} ATPase. In one series of experiments, the heart membranes were incubated with varying concentrations of CaATP (Ca^{++}/ATP = 1/1) for 10 min and the results concerning the ATP hydrolysis are shown in Fig. 11. Lineweaver-Burk analysis of these results gave a K_m of 0.90 ± 0.08 mM for CaATP and a V_{max} of 17.2 ± 1.7 μmoles Pi/mg per h. In another series, this membrane fraction was incubated in the presence of 4 mM ATP but in the presence of various concentrations of divalent cations, all of which were capable of stimulating ATP hydrolysis (Fig. 12). Over the concentration range employed in this study, both Mg^{++} and Mn^{++} stimulated ATP hydrolysis similar to Ca^{++}. However, Co^{++} stimulated up to

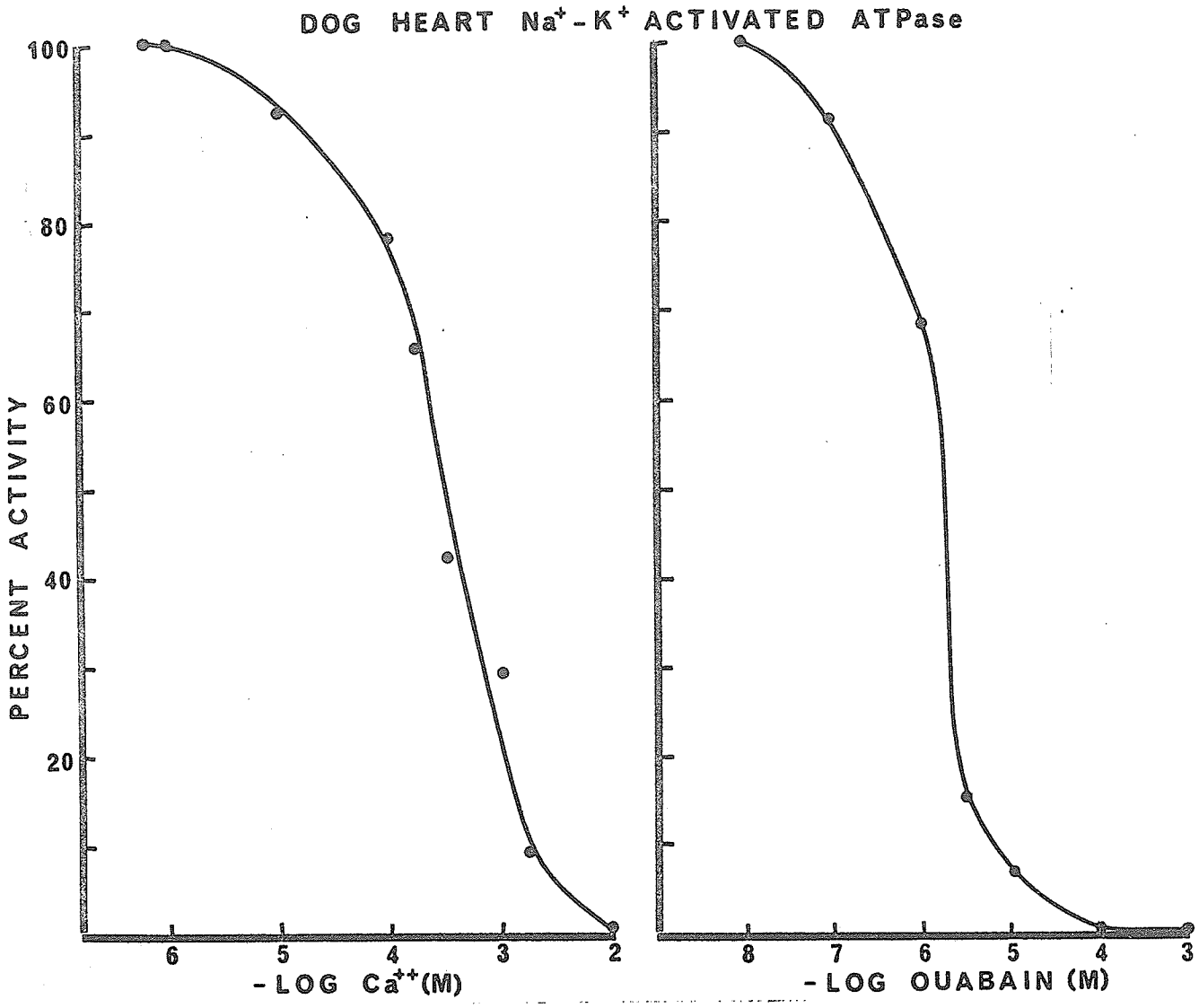

FIGURE 9 Effect of different concentrations of Ca^{++} or ouabain on the Na^{+}- K^{+}ATPase activity of the dog heart solubilized enzyme preparation. Conditions of incubation were the same as in Methods. Each value is an average of 5 experiments.

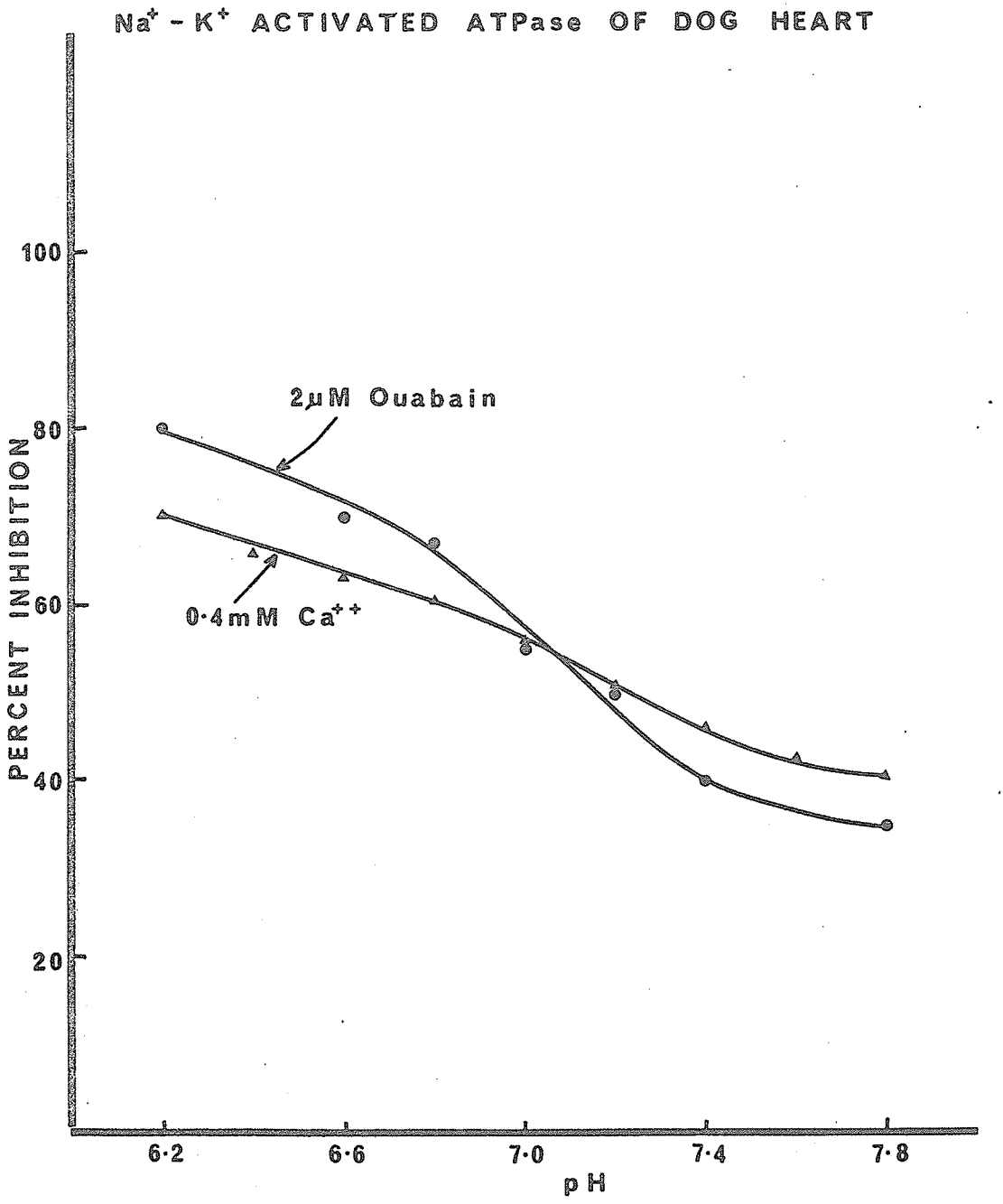

FIGURE 10 Influence of pH on the Ca^{++} or ouabain induced inhibition of the Na^{+}- K^{+}ATPase activity of the dog heart solubilized enzyme preparation. Conditions of incubation were the same as in Methods. Each value is an average of 3 experiments.

TABLE VIII

SOME PROPERTIES OF DOG HEART Na^+ - K^+ ATPase PREPARATIONS OBTAINED BY DIFFERENT PROCEDURES

The kinetic parameters shown were obtained by varying the substrate, ion or inhibitor in the incubation medium and applying Lineweaver-Burk analysis to the data thus obtained. The conditions of incubation were the same as for Figs 6 - 9. Each value is a mean ± S.E. of 3 - 5 experiments.

Parameter	Membrane fraction	NaI treated membrane fraction	Solubilized enzyme preparation
K_m (MgATP), mM	0.75 ± 0.12	0.70 ± 0.08	0.31 ± 0.07
Concn of K^+ for half maximal activation, mM	1.20 ± 0.32	1.50 ± 0.17	2.70 ± 0.46
Concn of Na^+ for half maximal activation, mM	12.00 ± 1.70	16.60 ± 2.10	20.20 ± 2.50
Concn of ouabain for 50% inhibition, μM	3.20 ± 0.53	3.10 ± 0.44	2.40 ± 0.27
Concn of calcium for 50% inhibition, mM	1.20 ± 0.23	1.00 ± 0.16	0.51 ± 0.12

TABLE IX

EFFECT OF VARIOUS SYMPATHOMIMETIC AGENTS AND CYCLIC AMP ON DOG HEART Na^+- K^+ATPase PREPARATIONS OBTAINED BY DIFFERENT PROCEDURES

Results are expressed as μmoles Pi/mg protein per h. All the agents were in the range of 50 - 150 μM and were added to the incubation medium immediately before the incubation began. The assay system was the same as in Methods. Each value is a mean ± S.E. of 8 experiments.

Agent	Membrane fraction	NaI treated membrane fraction	Solubilized enzyme preparation
Control	9.7 ± 0.5	15.4 ± 0.8	49.3 ± 2.3
Norepinephrine	10.2 ± 0.7	15.4 ± 0.7	47.2 ± 1.9
Isoproterenol	9.9 ± 0.7	15.2 ± 0.5	50.1 ± 2.7
Tyramine	9.7 ± 0.8	15.0 ± 0.8	49.3 ± 2.5
Metaraminol	9.0 ± 0.6	15.2 ± 0.8	48.0 ± 2.2
Ephedrine	9.8 ± 0.8	15.6 ± 0.9	48.7 ± 2.4
Nicotine	10.3 ± 0.5	15.4 ± 0.6	49.0 ± 2.4
Cyclic AMP	9.8 ± 0.8	15.1 ± 0.7	48.6 ± 2.1

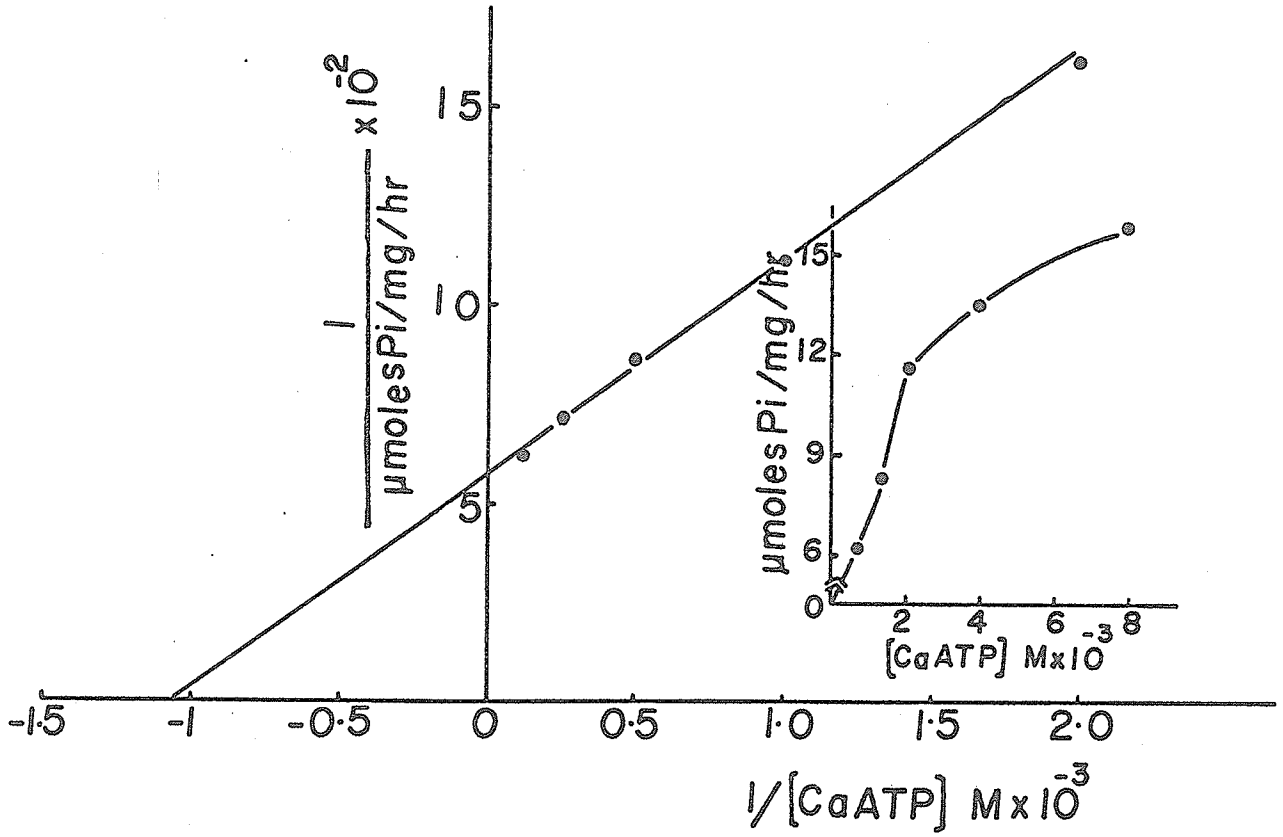

FIGURE 11 Effect of CaATP concentrations on the dog heart membrane Ca^{++}ATPase activity. The assay system was the same as in Methods except that the CaATP concentration was varied as indicated. The results are typical of 4 experiments.

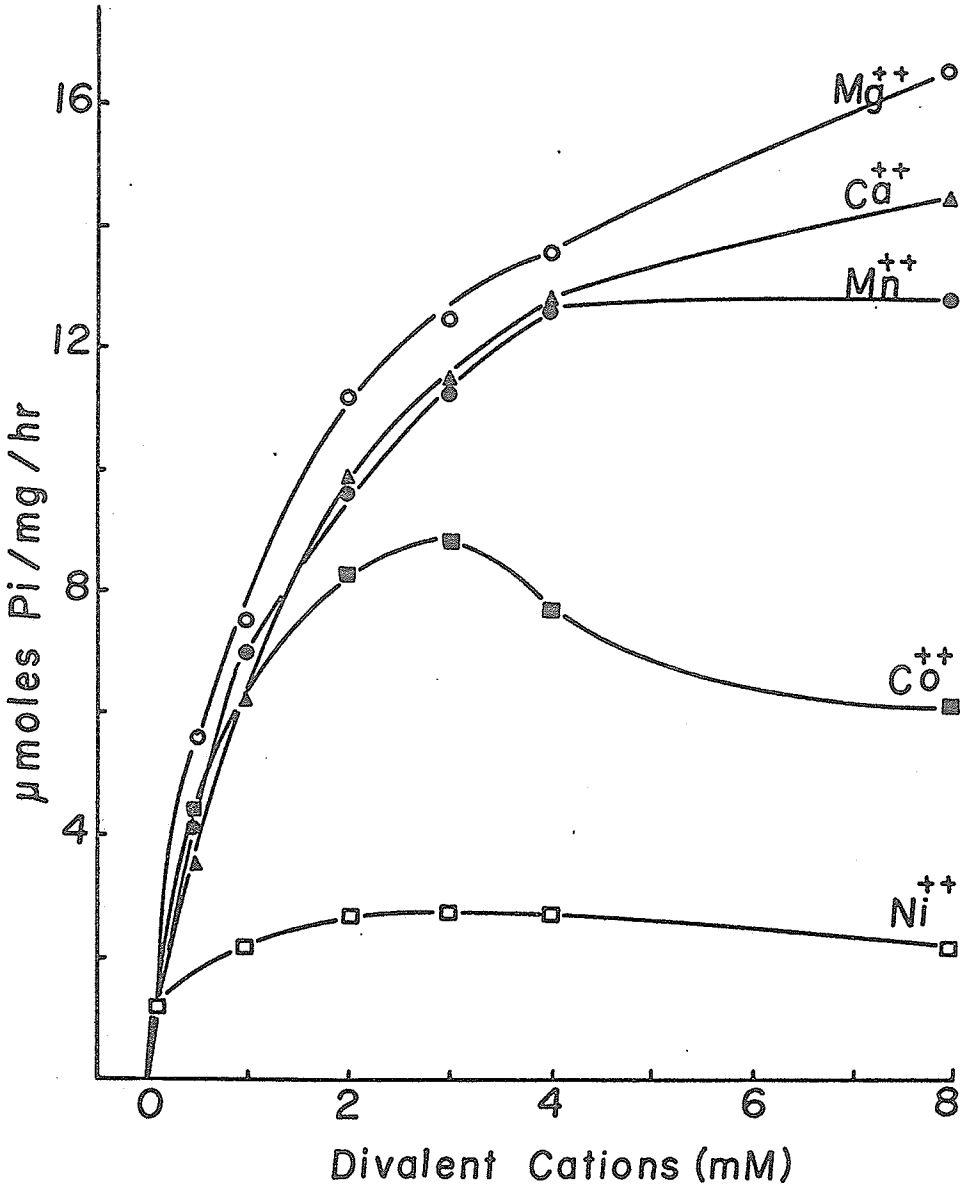

FIGURE 12 Effects of divalent cations on ATP hydrolysis by dog heart membranes. The assay system was the same as in Methods except that the concentrations of different cations, Mg^{++} (o—o), Ca^{++} (▲—▲), Mn^{++} (●—●), Co^{++} (■—■), and Ni^{++} (□—□) were varied. The results are the average of 5 experiments.

3 mM and thereafter began to depress ATP hydrolysis. Ni^{++} showed relatively less stimulation of ATP hydrolysis in comparison to the other divalent cations studied, showing maximal activation at about 2 mM. Excess of ATP in the incubation medium, beyond the concentration of the divalent cation, was found to inhibit ($P < 0.05$) the ATP hydrolysis due to Ca^{++}, Mg^{++}, Mn^{++}, Co^{++} and Ni^{++} (Table X).

In another series of experiments the effect of various divalent cations such as Mg^{++}, Mn^{++}, Co^{++} and Ni^{++} was studied on the ATP hydrolysis by heart membrane in the presence of Ca^{++}. All these cations were found to markedly inhibit ATP hydrolysis in the presence of 8 mM Ca^{++} (Fig. 13). The order of potency for the inhibition of Ca^{++} stimulated ATPase was $Ni^{++} > Co^{++} > Mg^{++} > Mn^{++}$. When the effect of these ions on ATP hydrolysis by the heart membrane was studied in the presence of 4 mM Ca^{++}, only Co^{++} and Ni^{++} were found to be inhibitory over the concentration range studied (Fig. 14).

The ATP hydrolysis due to Ca^{++} by the heart membrane fraction was also studied in the presence of varying concentrations of Na^{+} or K^{+} in the incubation medium. Na^{+} in concentrations from 10 - 20 mM was found to inhibit the ATP hydrolysis due to Ca^{++}; whereas, K^{+} in these concentrations was found to have no appreciable ($P > 0.05$) effect (Table XI). The data in Table XII show no action of various sympathomimetic agents and cyclic AMP on Ca^{++} stimulated ATPase activity of the heart membrane fraction.

In another set of experiments, the dog heart membranes were incubated with varying concentrations of MgATP and the results for ATP hydrolysis are shown

TABLE X

EFFECT OF ATP ON THE ATP HYDROLYSIS DUE TO DIFFERENT CATIONS BY DOG HEART MEMBRANES

Results are shown as μmoles Pi/mg protein per min. Conditions of incubation are the same as for Ca^{++}ATPase activity in the Methods, except that the concentrations of divalent cations and ATP were used as indicated. Each value is a mean ± S.E. of 6 experiments.

Cation	0.1 mM Cation		1 mM Cation	
	0.1 mM ATP	4 mM ATP	1 mM ATP	4 mM ATP
Calcium	4.5 ± 0.9	1.6 ± 0.2	9.7 ± 1.0	6.0 ± 0.2
Magnesium	4.9 ± 0.7	0.9 ± 0.2	12.0 ± 1.3	7.5 ± 0.6
Manganese	5.3 ± 0.9	2.0 ± 0.6	10.8 ± 1.1	7.0 ± 1.8
Cobalt	4.5 ± 0.5	2.6 ± 0.4	9.7 ± 1.3	6.2 ± 0.7
Nickle	3.4 ± 0.5	1.7 ± 0.3	3.9 ± 0.5	2.2 ± 0.2

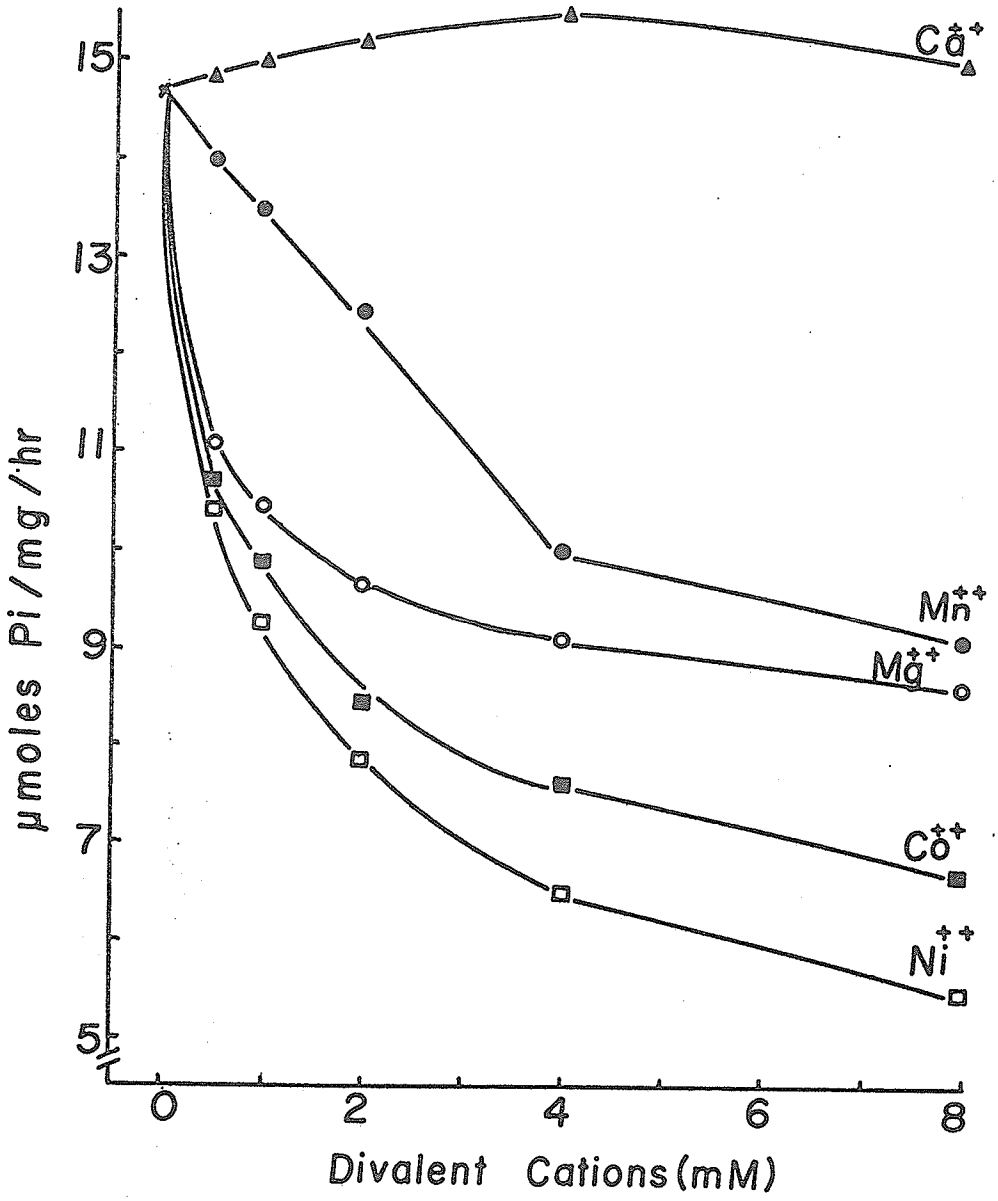

FIGURE 13 Effect of divalent cations on ATP hydrolysis by dog heart membranes in the presence of 8 mM Ca^{++}. The assay system was the same as in the Methods except that varying concentrations of different cations, Ca^{++} (◀—▲), Mn^{++} (●—●), Mg^{++} (○—○), Co^{++} (■—■), and Ni^{++} (□—□) were also present in addition to 8 mM Ca^{++}.

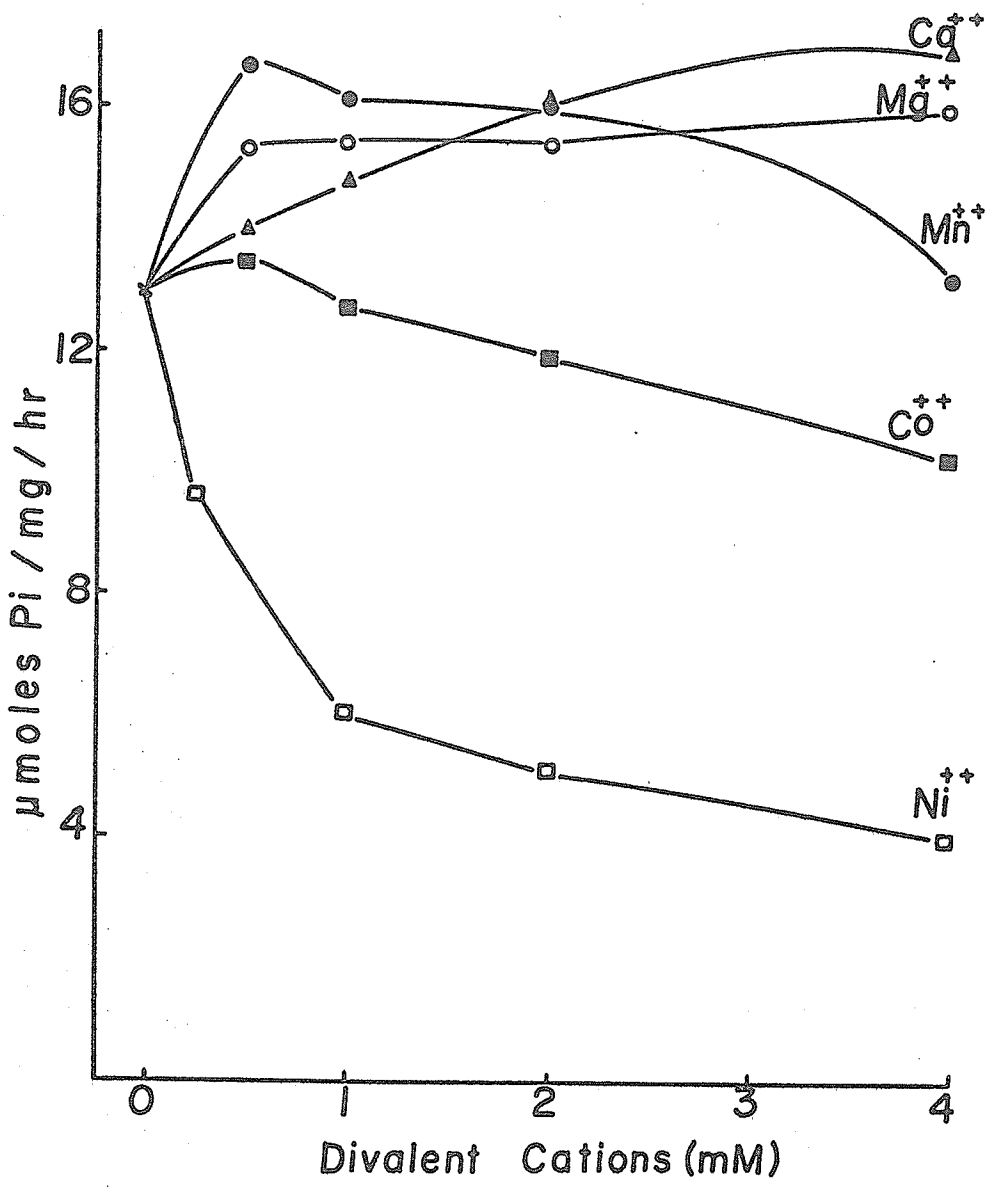

FIGURE 14 Effect of divalent cations on ATP hydrolysis by dog heart membranes in the presence of 4 mM Ca^{++}. The assay system was the same as in Methods except that varying concentrations of different cations, Ca^{++} (▲—▲), Mg^{++} (○—○), Mn^{++} (●—●), Co^{++} (■—■), and Ni^{++} (□—□) were also present in addition to 4 mM Ca^{++}. The results are the average of 4 experiments.

TABLE XI

EFFECT OF Na^+ AND K^+ ON THE ATP HYDROLYSIS DUE TO Ca^{++} BY DOG HEART MEMBRANES

Results are expressed as μmoles Pi/mg protein per min. The assay system was the same as for Ca^{++}ATPase activity in the Methods, except for the concentration of Na^+ and K^+ as indicated. Each value is a mean ± S.E. of 6 experiments.

Concn of Na^+ or K^+ (mM)	4 mM Ca^{++}	8 mM Ca^{++}
-	12.6 ± 0.6	14.5 ± 0.7
Na^+, 5	11.4 ± 0.5	13.0 ± 0.4
Na^+, 10	10.7 ± 0.5	11.6 ± 0.4
Na^+, 20	10.0 ± 0.4	10.3 ± 0.6
K^+, 5	12.8 ± 0.7	14.2 ± 1.2
K^+, 10	13.2 ± 1.5	14.8 ± 1.0
K^+, 20	13.5 ± 1.8	14.7 ± 1.1

TABLE XII

EFFECT OF VARIOUS SYMPATHOMIMETIC AGENTS ON Mg^{++}ATPase AND Ca^{++}ATPase ACTIVITIES OF DOG HEART MEMBRANES

The results are expressed as μmoles Pi/mg protein per min. The concentrations of the agents ranged from 50 - 150 μM. The assay systems were the same as in Methods. Each value is a mean ± S.E. of 4 - 6 experiments.

Agent	Ca^{++}ATPase	Mg^{++}ATPase
Control	11.6 ± 1.2	13.0 ± 1.1
Norepinephrine	11.2 ± 1.3	12.9 ± 1.6
Isoproterenol	11.8 ± 1.3	12.6 ± 1.5
Tyramine	10.8 ± 1.1	12.6 ± 1.4
Metaraminol	10.3 ± 1.1	12.2 ± 1.8
Ephedrine	11.6 ± 1.7	13.2 ± 1.3
Nicotine	12.7 ± 1.8	13.4 ± 1.6
Cyclic AMP	11.6 ± 1.7	11.9 ± 1.7

in Fig. 15. The values for K_m and V_{max} for Mg^{++}ATPase are 0.65 ± 0.10 mM and 16.0 ± 1.2 μmoles Pi/mg per h respectively. ATP hydrolysis due to Mg^{++} was also not affected by the presence of various sympathomimetic agents and cyclic AMP (Table XII).

The dog heart membranes were found to contain highly active adenylate cyclase, which was stimulated by epinephrine and NaF. The dose response for epinephrine and NaF are shown in Fig. 16. It can be seen that the maximal activation by epinephrine was about 35% and that by NaF was 4 fold of the basal levels. The activation due to epinephrine was found to be inhibited by a well known β-adrenergic blocking agent, propranolol (Table XIII). Both norepinephrine and isoproterenol were also found to stimulate adenylate cyclase of heart membranes while other sympathomimetic agents such as tyramine, metaraminol, ephedrine and nicotine had no appreciable effect (Table XIII). The adenylate cyclase activity in the absence and presence of epinephrine and NaF showed a broad pH optimum between 8.0 and 8.5 (Fig. 17). It can be seen from Fig. 18 that the activity of adenylate cyclase of the membrane fraction depended upon the concentration of Mg^{++} in the incubation medium and saturation was observed at about 8 mM. Both epinephrine and NaF increased the velocity of adenylate cyclase reaction at every concentration of Mg^{++} studied. The effect of increasing the concentration of ATP at a fixed concentration of Mg^{++} on the adenylate cyclase activity of the heart membrane in the absence and presence of epinephrine on NaF is shown in Fig. 19. The K_m for ATP was found to be 0.082 ± 0.007 mM and was not affected by either

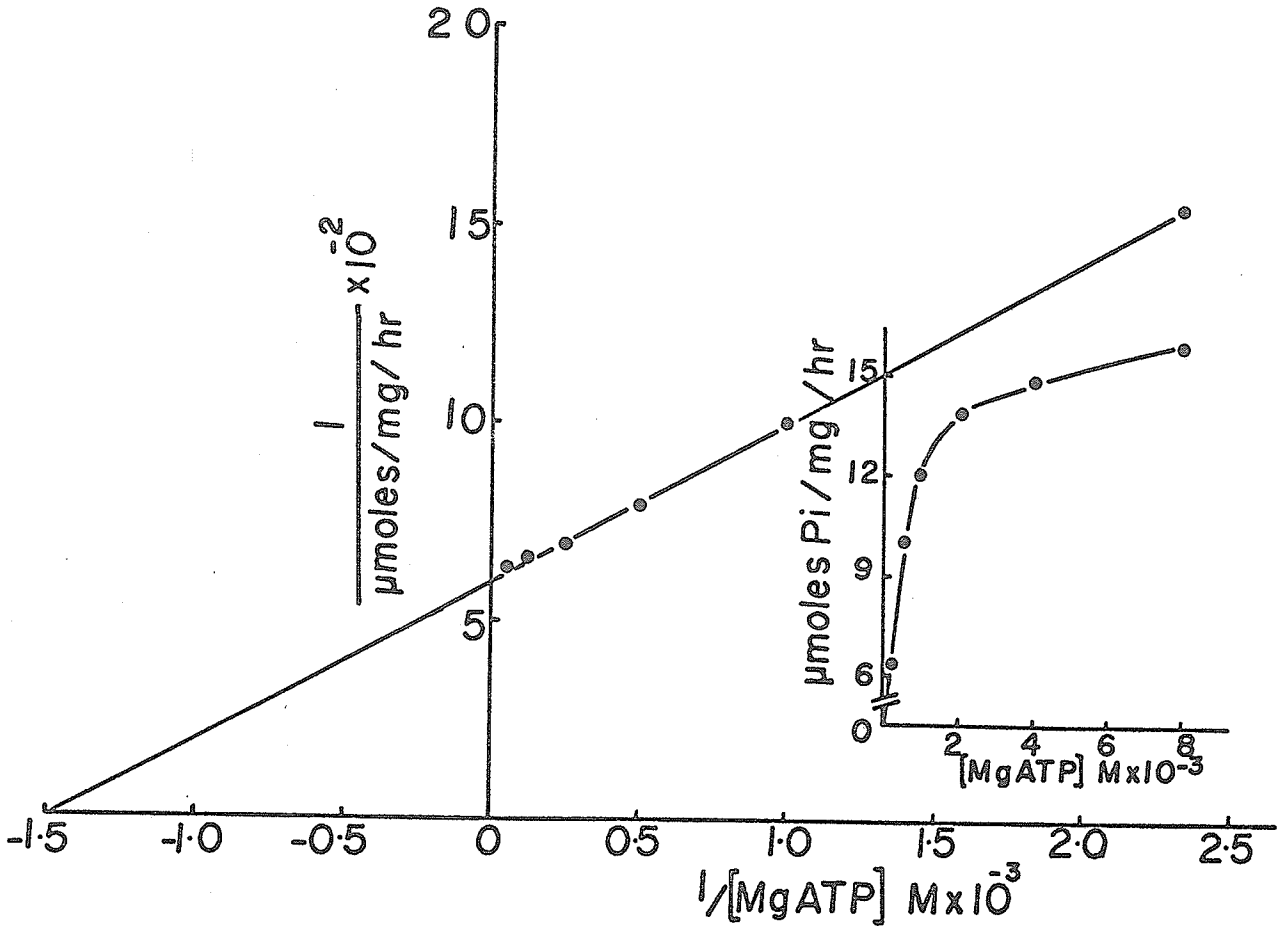

FIGURE 15 Effect of MgATP concentrations in the dog heart membrane Mg^{++}ATPase activity. The assay system was the same as in Methods except that the MgATP concentration was varied as indicated. The results are typical of 4 experiments.

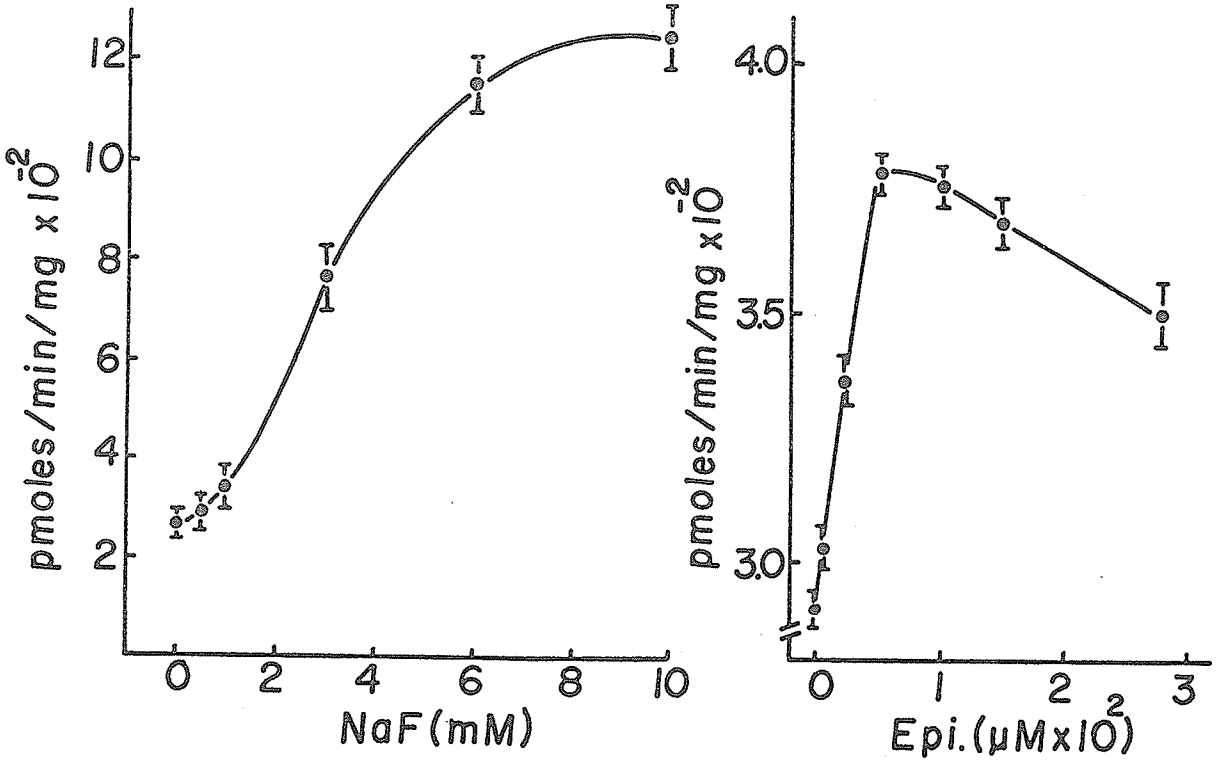

FIGURE 16 Activation of dog heart membrane adenylate cyclase by NaF and epinephrine. The assay system was the same as in Methods except that the activity was measured in the presence of different concentrations of NaF or epinephrine (Epi.). Each value is a mean ± S.E. of 6 experiments.

TABLE XIII

EFFECT OF VARIOUS SYMPATHOMIMETIC AGENTS ON ADENYLATE CYCLASE ACTIVITY OF DOG HEART MEMBRANE PREPARATION

The assay system was the same as in Methods. The concentration of each agent varied from 25 - 100 μM. Each value is a mean ± S.E. of 6 experiments.

Agent	Enzyme activity (pmoles cyclic AMP/mg per min)
Control	287 ± 18
Norepinephrine	368 ± 25
Isoproterenol	400 ± 27
Tyramine	278 ± 21
Metaraminol	301 ± 23
Ephedrine	298 ± 17
Nicotine	291 ± 17
Epinephrine	375 ± 21
Propranolol + epinephrine	271 ± 14

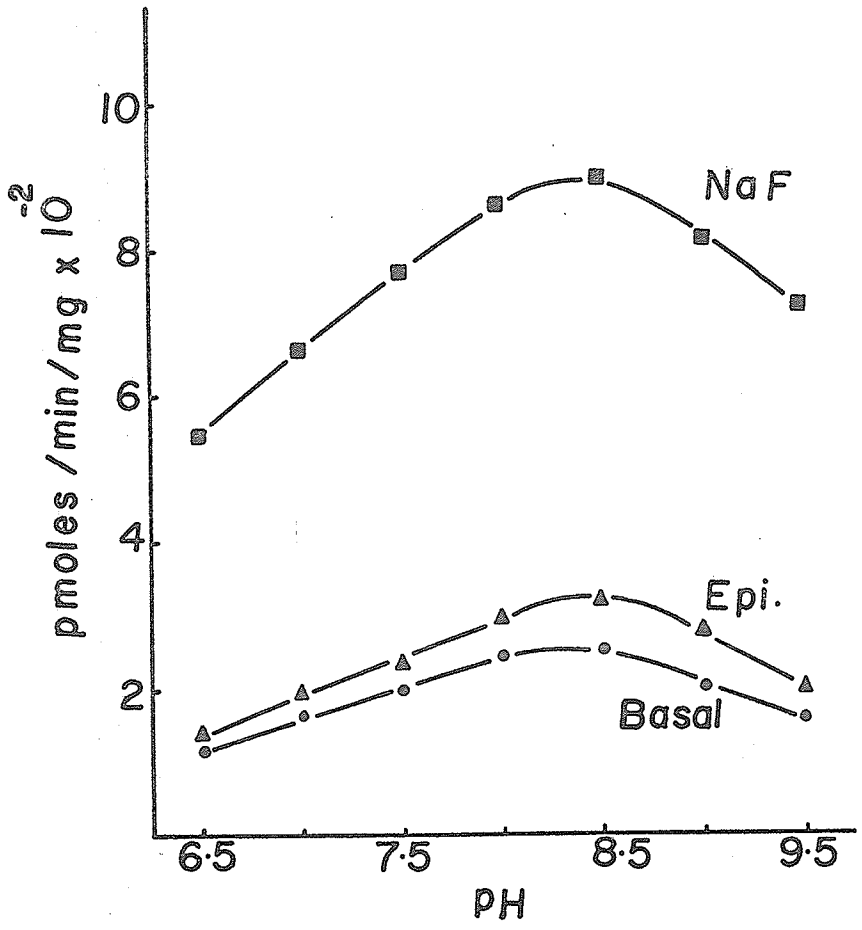

FIGURE 17 Effect of pH on the dog heart membrane adenylate cyclase activity in the absence (basal) and presence of NaF (5 mM) or epinephrine (25 μM). The assay system was the same as in Methods except for the presence of NaF or epinephrine (Epi.) as indicated. Each value is an average of 3 experiments.

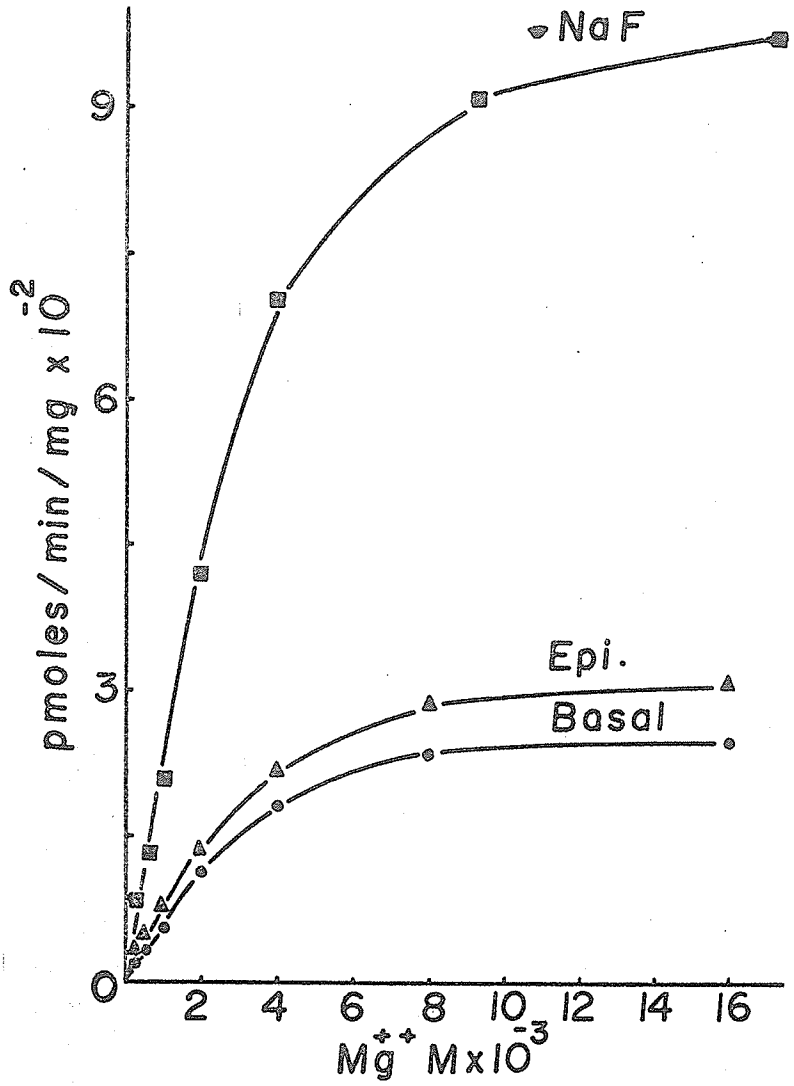

FIGURE 18 Effect of Mg^{++} on the dog heart membrane adenylate cyclase activity in the absence (basal) and presence of NaF (6 mM) or epinephrine (25 μM). The assay system was the same as in Methods except that the concentration of Mg^{++} was varied as indicated. Each value is an average of 3 experiments.

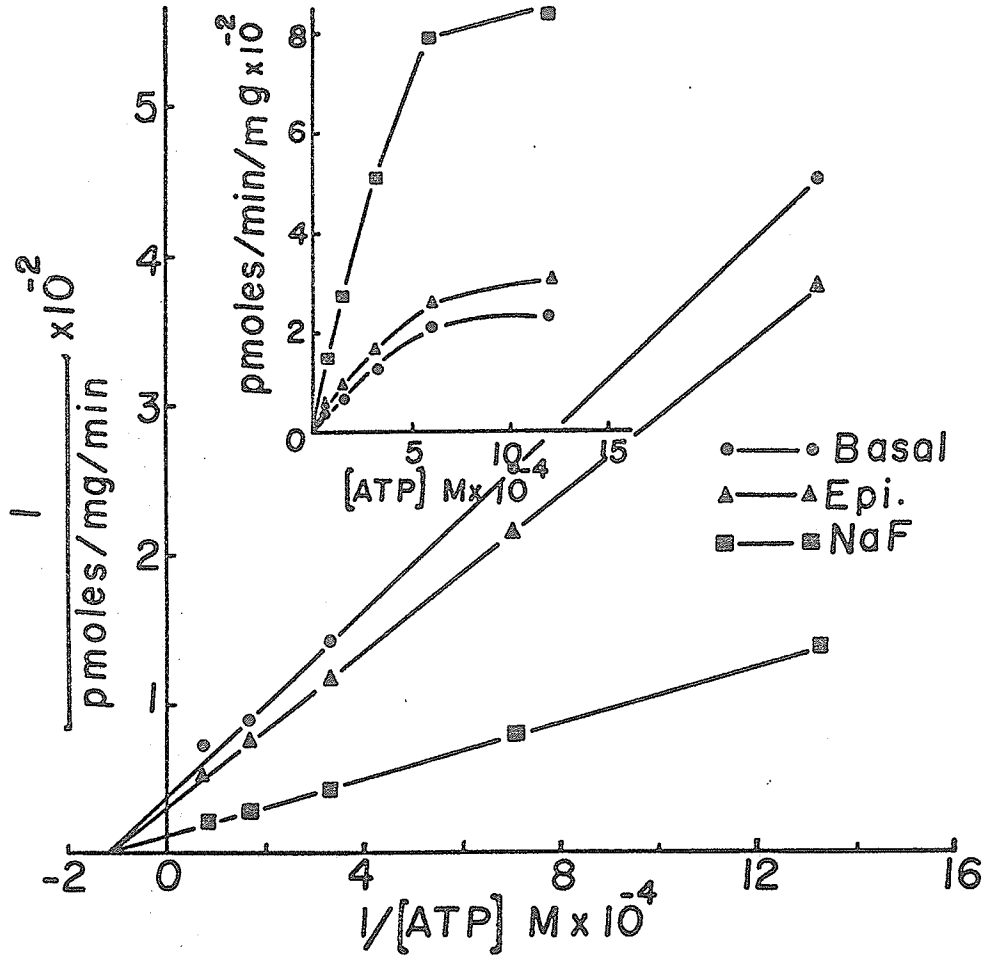

FIGURE 19 Effect of ATP concentration on the dog heart membrane adenylate cyclase in the absence (basal) and presence of NaF (6 mM) or epinephrine (25 μM). The assay system was the same as in Methods except that the concentration of ATP was varied as indicated. The results are typical of 6 experiments.

epinephrine or NaF. The value for V_{max} for basal activity was found to be 286 ± 21 pmoles cyclic AMP/mg per min; both epinephrine and NaF were found to increase the value for V_{max}.

4. Membrane Bound Enzyme Activities in Rat Hearts Perfused with Media in the Absence of Different Cations

Isolated rat hearts were perfused for 20 min with different aerobic media lacking Na^+, K^+, Mg^{++} or Ca^{++} and the membrane fraction was isolated in each case. The results concerning the activities of adenylate cyclase, Mg^{++}ATPase, Ca^{++}ATPase and $Na^+ - K^+$ATPase are shown in Table XIV. Perfusing the hearts with Na^+ free or K^+ free medium significantly ($P < 0.05$) decreased $Na^+ - K^+$ ATPase activity without any appreciable effects on the activities of adenylate cyclase, Mg^{++}ATPase or Ca^{++}ATPase. No significant ($P > 0.05$) change in the membrane bound enzyme activities was noted in preparations obtained from hearts perfused with Mg^{++} free medium. On the other hand, perfusion of hearts with Ca^{++} free medium for 20 min resulted in a significant ($P < 0.05$) decrease in the activities of adenylate cyclase, Mg^{++}ATPase and $Na^+ - K^+$ATPase without any significant effect on the Ca^{++}ATPase activity. It should be noted that the hearts perfused with medium in the absence of Ca^{++}, Na^+ or K^+ for 20 min were in complete cardiac arrest, whereas, those perfused with Mg^{++} free medium generated normal degree of contractile force.

In another series of experiments hearts were perfused with Ca^{++} free medium for varying intervals and adenylate cyclase activity of the membrane fraction was studied in the absence and presence of NaF. It can be seen from

TABLE XIV

CHANGES IN ENZYME ACTIVITIES OF RAT HEART PREPARATIONS AFTER 20 MINUTE PERFUSION WITH MEDIA IN THE ABSENCE OF DIFFERENT CATIONS

Results for adenylate cyclase are expressed as pmoles cyclic AMP formed/mg protein per min. Results for Mg^{++}ATPase, Ca^{++}ATPase and Na^{+}- K^{+} ATPase are shown as μmoles Pi/mg protein per h. Rat hearts were perfused for 20 min with media in the absence of the indicated cation, the membrane fraction was isolated, and the assays for adenylate cyclase, Mg^{++}ATPase, Ca^{++}ATPase and Na^{+}- K^{+}ATPase were carried out as described in Methods. Each value is a mean ± S.E. of 6 - 8 experiments.

Conditions	Adenylate cyclase	Mg^{++} ATPase	Ca^{++} ATPase	Na^{+}- K^{+} ATPase
Control	370 ± 25	25.8 ± 2.1	31.3 ± 2.1	11.2 ± 1.0
Na^{+} free perfusion	339 ± 21	30.1 ± 1.8	35.2 ± 2.4	6.3 ± 0.6
K^{+} free perfusion	334 ± 16	23.5 ± 2.0	28.7 ± 0.9	7.7 ± 0.3
Mg^{++} free perfusion	356 ± 19	24.2 ± 1.5	30.2 ± 1.6	10.9 ± 1.1
Ca^{++} free perfusion	271 ± 18	19.1 ± 1.2	27.5 ± 1.0	7.5 ± 0.8

Table XV that the yield of the membrane protein did not alter significantly ($P > 0.05$) upon perfusing the hearts over varying intervals; whereas, a significant depression in basal adenylate cyclase activity became apparent at 10 min of perfusion. The stimulatory effect of NaF on membrane adenylate cyclase activity was enhanced ($P < 0.05$) after 10 - 20 min of perfusion with Ca^{++} free medium. The adenylate cyclase activities of the membrane fraction obtained from rat hearts perfused with control or Ca^{++} free medium for 20 min was linear with respect to time of incubation (Fig. 20); the activity being significantly lower ($P < 0.05$) at each time interval in preparations obtained from hearts perfused with Ca^{++} free medium. The stimulation of adenylate cyclase by various concentrations of NaF in membrane fractions obtained from hearts perfused with Ca^{++} free medium for 20 min was greater ($P < 0.05$) than the control values at every concentration of NaF employed in this study (Fig. 21).

The time course of changes in Mg^{++}ATPase, Ca^{++}ATPase and $Na^{+} - K^{+}$ ATPase activities of the rat heart membranes was also investigated on perfusion with Ca^{++} free medium for different intervals. The results in Table XVI show a significant ($P < 0.05$) decrease in Mg^{++}ATPase and $Na^{+} - K^{+}$ATPase occurred at 10 min without any appreciable changes in the activity of Ca^{++}ATPase. The activities of Mg^{++}ATPase (Fig. 22) and $Na^{+} - K^{+}$ATPase (Fig. 23) when determined in the presence of different concentrations of MgATP ($Mg^{++}/ATP = 1/1$) were lower ($P < 0.05$) at each point. On the other hand, Ca^{++}ATPase activities of the preparations obtained from hearts perfused with control and Ca^{++} free medium for 20 min were not different from each other ($P > 0.01$) when determined

TABLE XV

TIME COURSE OF CHANGES IN PROTEIN YIELD AND ADENYLATE CYCLASE ACTIVITY OF RAT HEART SARCOLEMMA ON PERFUSION WITH Ca^{++} FREE MEDIUM

Each value is a mean ± S.E. of 4 experiments. The assay system was the same as in Methods.

Time of perfusion (min)	Protein yield (mg/g tissue)	Basal adenylate cyclase activity (pmoles cyclic AMP/mg per min)	Stimulation of adenylate cyclase activity by 1 mM NaF (% of basal)
Control	9.6 ± 0.8	375 ± 20	38 ± 3
1	9.3 ± 0.4	361 ± 11	39 ± 2
5	9.1 ± 0.5	307 ± 9	45 ± 2
10	8.7 ± 0.4	273 ± 11	56 ± 4
20	8.9 ± 0.6	265 ± 15	54 ± 3

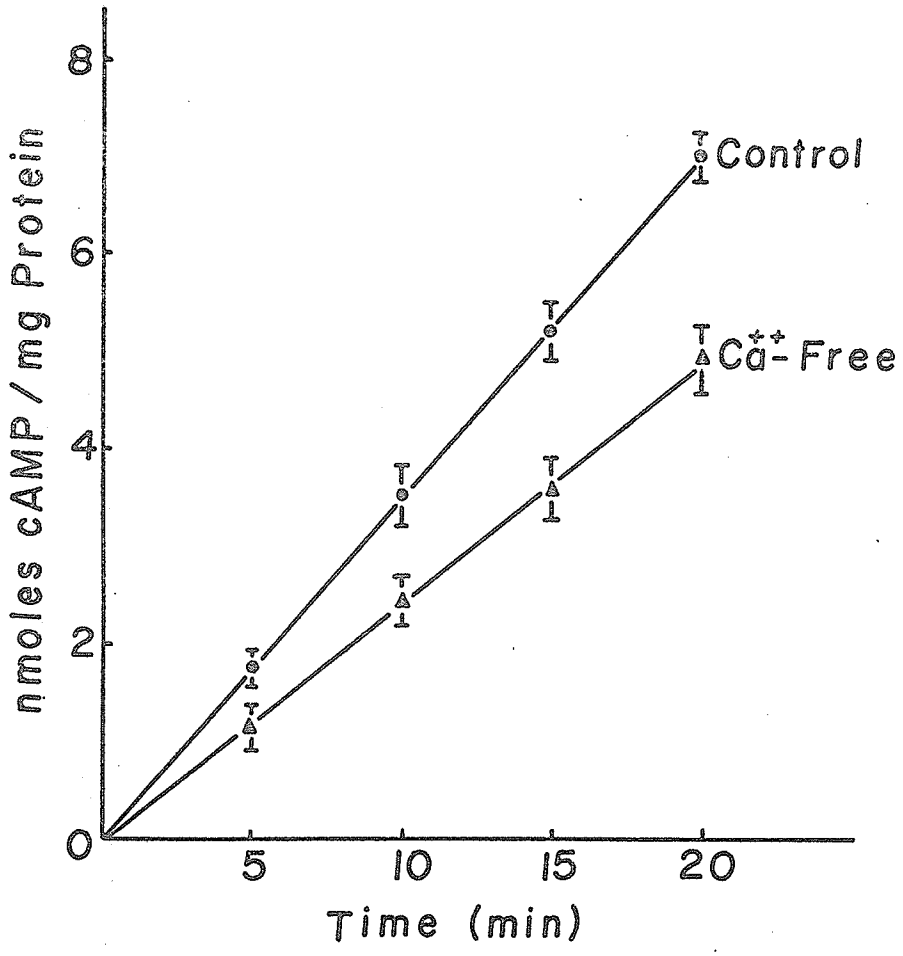

FIGURE 20 Adenylate cyclase activity at different times of incubation of the membrane preparations from rat hearts perfused for 20 min with control or Ca^{++} free medium. The assay system was the same as in Methods except that the incubation was carried out for different intervals as indicated. Each value is a mean ± S.E. of 6 experiments.

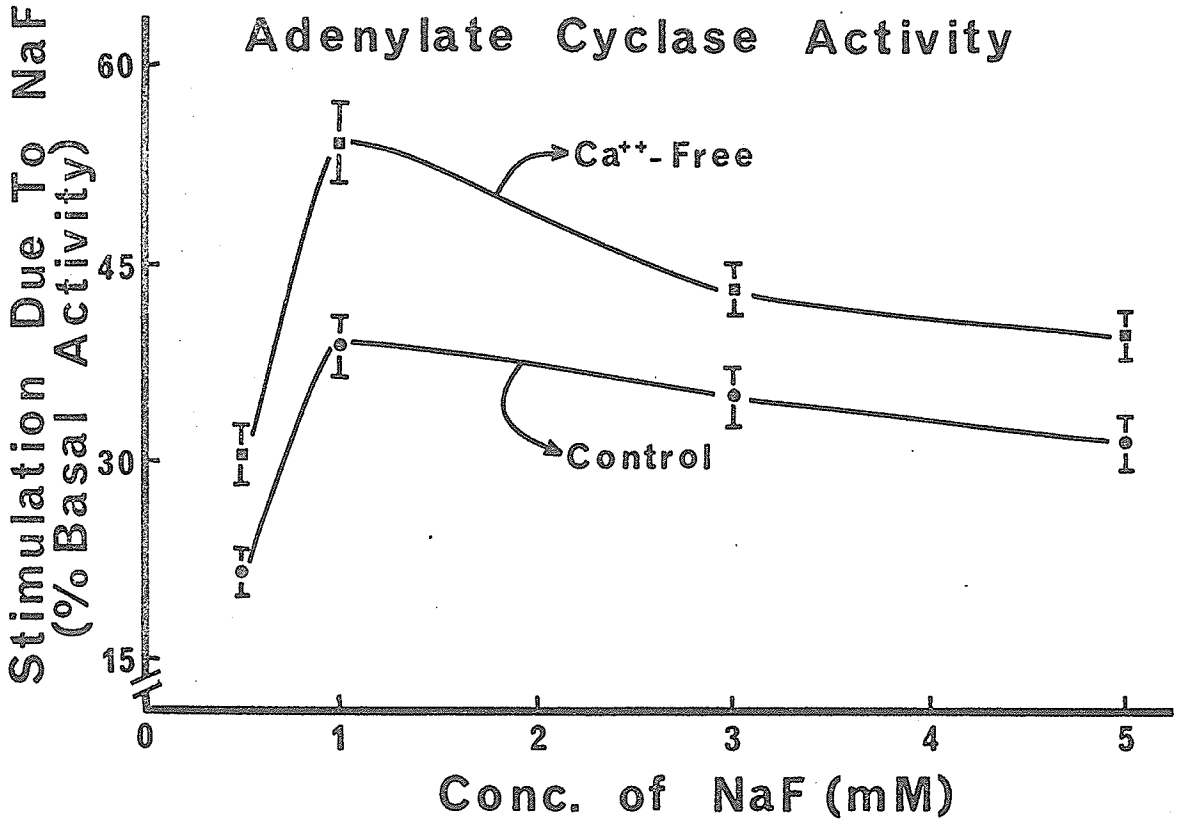

FIGURE 21 NaF stimulation of the membrane adenylate cyclase from rat hearts perfused for 20 min with control or Ca^{++} free medium. The assay system was the same as in Methods except that the enzyme activity was determined in the presence of different concentrations of NaF. The values for the basal activities for the control and Ca^{++} free perfused hearts were similar to those described in Table XV. Each value is a mean ± S.E. of 6 experiments.

TABLE XVI

TIME COURSE OF CHANGES IN Mg^{++}ATPase, Na^{+} - K^{+}ATPase AND Ca^{++}ATPase OF RAT HEART SARCOLEMMA ON PERFUSION WITH Ca^{++} FREE MEDIUM

Each value is a mean ± S.E. of 4 experiments. The assay systems are the same as in Methods.

Time of perfusion (min)	ATPase activities (μmoles Pi/mg protein per h)		
	Mg^{++}ATPase	Ouabain sensitive Na^{+}- K^{+}ATPase	Ca^{++}ATPase
Control	25.2 ± 1.3	11.1 ± 0.55	29.2 ± 1.9
1	24.9 ± 1.4	12.0 ± 0.62	27.8 ± 1.7
5	23.6 ± 1.1	10.3 ± 0.44	28.4 ± 1.5
10	20.7 ± 1.0	9.1 ± 0.32	27.6 ± 1.7
20	18.8 ± 0.8	8.0 ± 0.56	27.4 ± 1.6

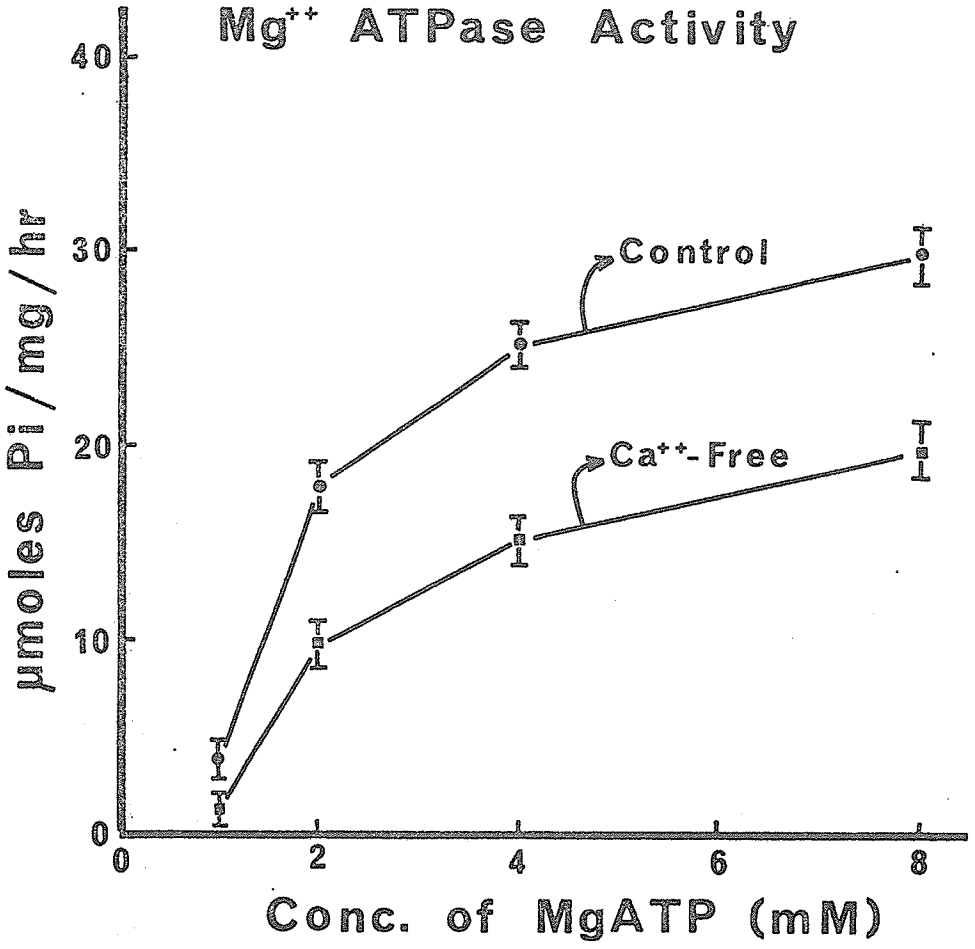

FIGURE 22 Membrane Mg^{++}ATPase activity of rat hearts perfused for 20 min with control or Ca^{++} free medium. The assay system was the same as in Methods except that the concentration of MgATP was varied as indicated. Each value is a mean ± S.E. of 6 experiments.

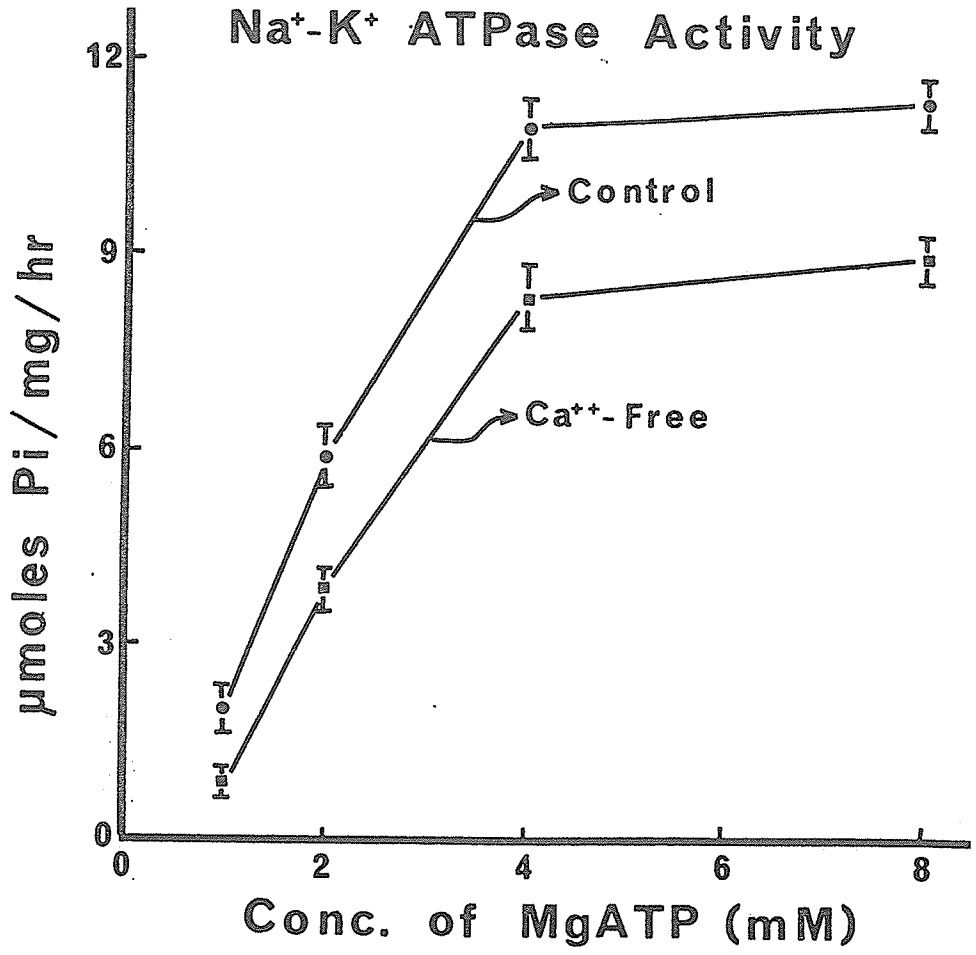

FIGURE 23

Membrane $Na^+ - K^+$ ATPase activity of rat hearts perfused for 20 min with control or Ca^{++} free medium. The assay system was the same as in Methods except that the concentration of MgATP was varied as indicated. Each value is a mean $\pm$ S.E. of 6 experiments.

at varying concentrations of CaATP (Ca^{++}/ATP = 1/1) in the incubation medium (Fig. 24).

In order to examine whether or not the changes in membrane bound enzyme activities due to Ca^{++} free perfusion were reversible, the rat hearts preperfused with Ca^{++} free medium were further perfused with control medium containing 1.25 mM Ca^{++} for 10 min. The membranes from these hearts were isolated and the activities of the membrane bound enzymes are shown in Table XVII. The basal adenylate cyclase activity was further decreased ($P < 0.05$) and the stimulatory effect of NaF was lost on preparations obtained from hearts further perfused with medium containing Ca^{++}. A marked decrease in Mg^{++}ATPase, Ca^{++}ATPase and Na^{+} - K^{+}ATPase activities was also noted in these preparations.

5. Membrane Bound Enzyme Activities in Failing Myopathic Hamster Hearts

All the myopathic hamsters employed in this study exhibited a severe degree of heart failure as evidenced from generalized edema, pulmonary edema, liver congestion and cardiac hypertrophy. The heart and liver weights were 30 - 40% greater than those of the controls.

The adenylate cyclase activities of the heart membrane fraction were determined in the absence and presence of epinephrine and NaF and the results are shown in Tables XVIII and XIX. The basal adenylate cyclase activity of the failing heart membranes was not different from the control ($P > 0.05$). However, the dose response study, for both epinephrine and NaF, revealed that the activation of adenylate cyclase in the failing heart preparations due to these agents was markedly reduced.

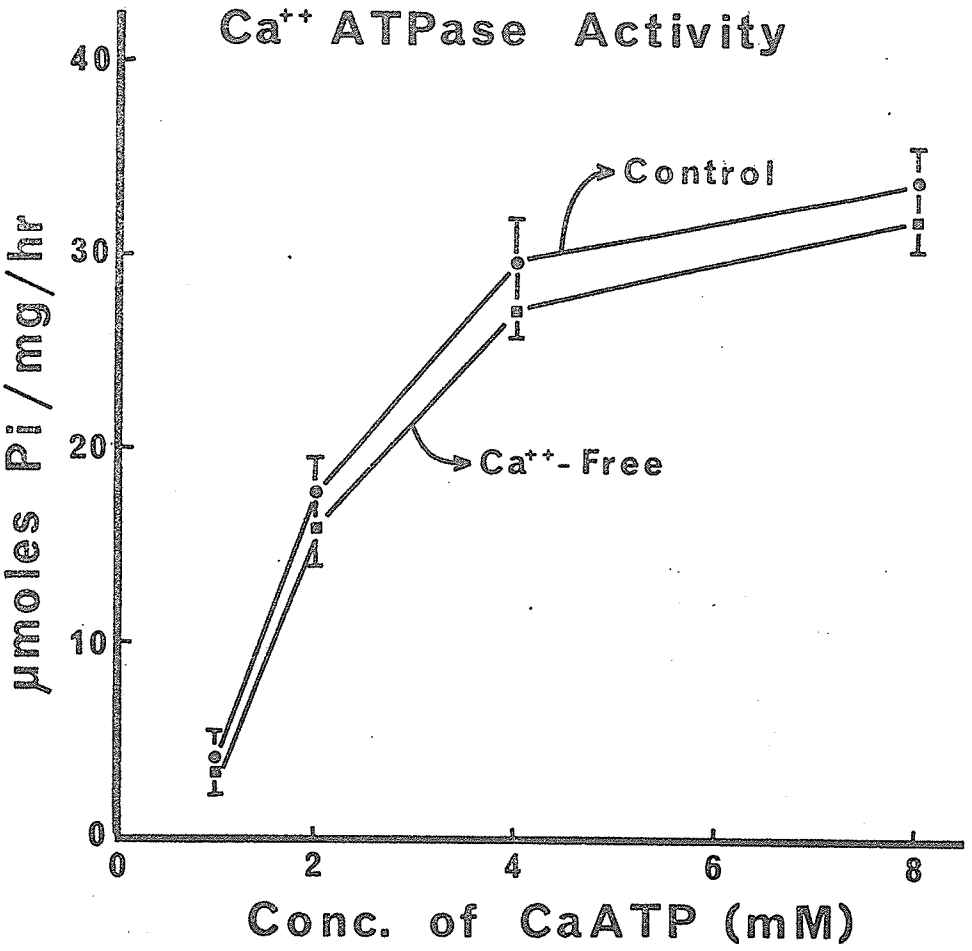

FIGURE 24 Membrane Ca^{++}ATPase activity of rat hearts perfused for 20 min with control or Ca^{++} free medium. The assay system was the same as in Methods except that the concentration of CaATP was varied as indicated. Each value is a mean ± S.E. of 6 experiments.

TABLE XVII

EFFECT OF PERFUSION WITH MEDIUM CONTAINING 1.25 mM CALCIUM ON ENZYME ACTIVITIES OF PREPARATIONS OBTAINED FROM HEARTS PREPERFUSED WITH CALCIUM FREE MEDIUM FOR 20 MINUTES

Adenylate cyclase activity is expressed as pmoles cyclic AMP formed/mg protein per min. Mg^{++}ATPase, Ca^{++}ATPase and Na^{+}- K^{+}ATPase activities are expressed as μmoles Pi/mg protein per h. The assay systems were the same as in Methods. Each value is a mean ± S.E. of 4 - 6 experiments.

Conditions	Adenylate cyclase		Mg^{++} ATPase	Ca^{++} ATPase	Na^{+}- K^{+} ATPase
	Basal	In the presence of 1 mM NaF			
Control	362 ± 27	507 ± 36	25.0 ± 1.5	29.6 ± 1.2	10.5 ± 0.6
Ca^{++} free perfusion	251 ± 23	387 ± 28	18.8 ± 1.0	27.0 ± 1.7	7.0 ± 0.5
Ca^{++} free perfusion followed by perfusion with Ca^{++} medium for 10 min	96 ± 6	80 ± 7	12.3 ± 2.2	23.2 ± 0.5	3.0 ± 0.7

TABLE XVIII

ADENYLATE CYCLASE ACTIVITY OF CONTROL AND FAILING HAMSTER HEART MEMBRANES IN THE ABSENCE OR PRESENCE OF EPINEPHRINE

Results are expressed as pmoles cyclic AMP formed/mg protein per min. Epinephrine was added to the incubation medium immediately before incubation began. The assay system was the same as in Methods. Failing hearts were obtained from cardiomyopathic hamsters (UM-X7.1) at advanced degree of congestive heart failure. Each value is a mean ± S.E. of 5 - 8 experiments.

Concentration of epinephrine (μM)	Control heart	Failing heart
-	225 ± 18	230 ± 21
50	300 ± 23	243 ± 15
100	327 ± 20	256 ± 26
150	321 ± 27	251 ± 19

TABLE XIX

ADENYLATE CYCLASE ACTIVITY OF CONTROL AND FAILING HAMSTER HEART MEMBRANES IN THE ABSENCE OR PRESENCE OF NaF

Results are expressed as pmoles cyclic AMP formed/mg protein per min. The assay system was the same as in Methods except that the concentrations of NaF were varied. Failing hearts were obtained from cardiomyopathic hamsters (UM-X7.1) at advanced degree of congestive heart failure. Each value is a mean ± S.E. of 5 - 8 experiments.

Concentration of NaF (mM)	Control heart	Failing heart
-	231 ± 17	229 ± 15
0.5	342 ± 28	258 ± 14
1.0	525 ± 31	359 ± 36
2.0	575 ± 34	376 ± 28

In another series of experiments, the activities of Ca^{++}ATPase, Mg^{++}ATPase and Na^{+} - K^{+}ATPase of the control and failing heart membranes were studied. The determination of Ca^{++}ATPase activity was carried out at varying concentrations of CaATP, whereas, those of Mg^{++}ATPase and Na^{+} - K^{+} ATPase were done at varying concentrations of MgATP in the incubation medium and the results are shown in Figs 25 - 27. The activities of Ca^{++}ATPase, Mg^{++} ATPase and Na^{+} - K^{+}ATPase in the membranes prepared from failing hearts were significantly depressed ($P < 0.05$) at every point in comparison to the control values.

In order to test whether or not the observed decrease in enzyme activities were due to contaminating non-sarcolemmal proteins, the membranes were prepared from homogenates in the absence or presence of exogenously added mitochondria or microsomes obtained from the failing hearts. The results shown in Table XX show that the activities of Ca^{++}ATPase, Mg^{++}ATPase, Na^{+} - K^{+} ATPase and adenylate cyclase of membranes thusly prepared did not significantly differ ($P > 0.05$) from each other.

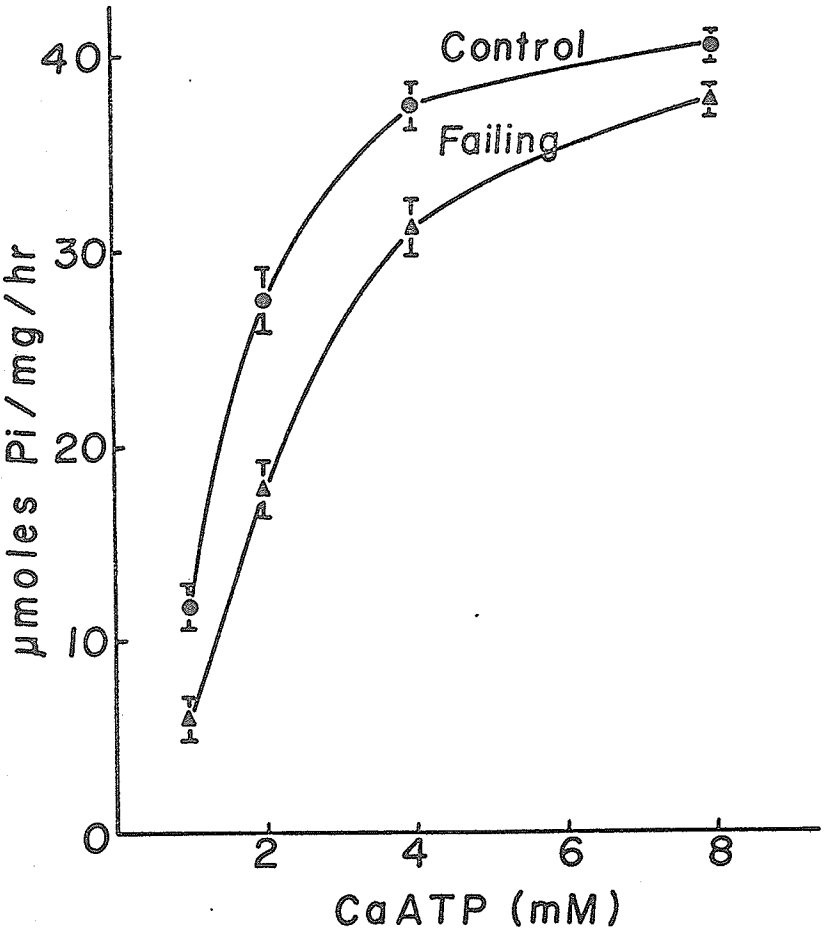

FIGURE 25 Ca^{++}ATPase activity at different concentrations of CaATP of the membrane preparations from control or cardiomyopathic (UM-X7.1) failing hamster hearts. The assay system was the same as in Methods except that the concentration of CaATP was varied as indicated. Each value is a mean ± S.E. of 7 - 10 experiments.

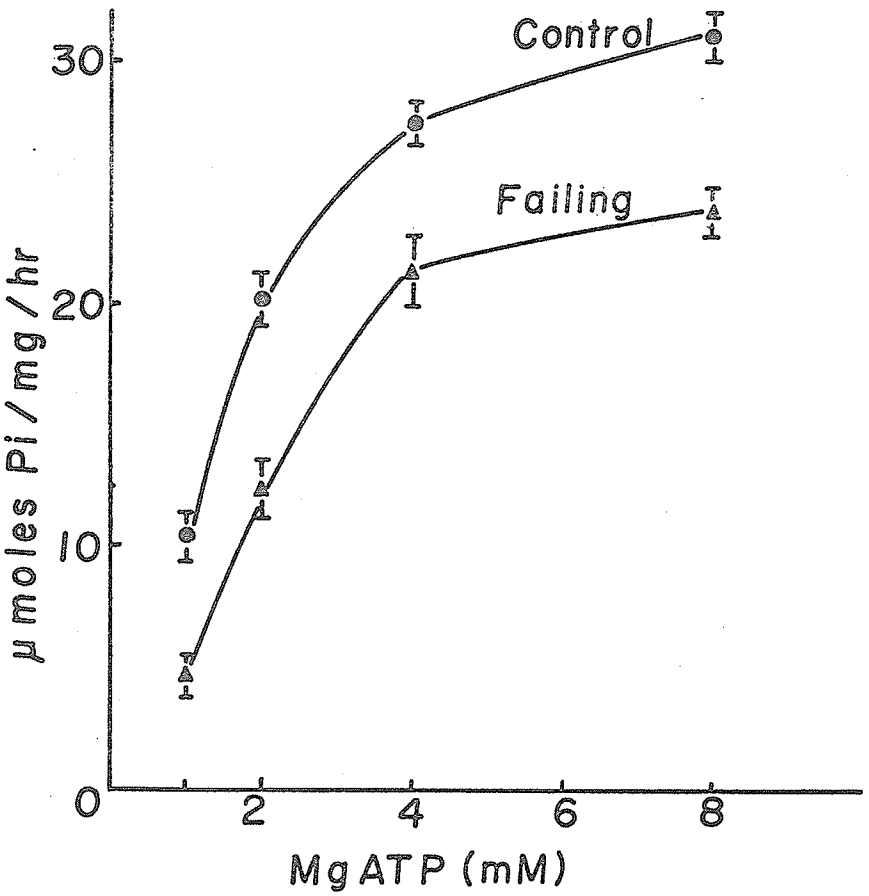

FIGURE 26 Mg^{++}ATPase activity at different concentrations of MgATP of the membrane preparations from control or cardiomyopathic (UM-X7.1) failing hamster hearts. The assay system was the same as in Methods except that the concentration of MgATP was varied as indicated. Each value is a mean ± S.E. of 7 - 10 experiments.

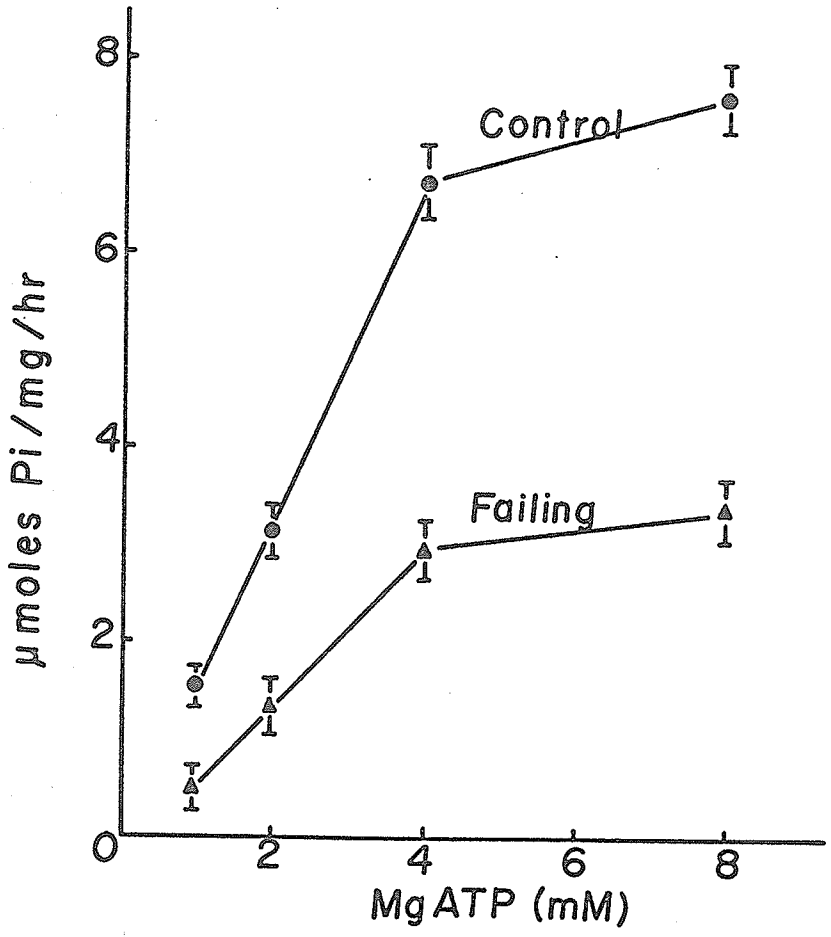

FIGURE 27 $Na^+ - K^+$ ATPase activity at different concentrations of MgATP of the membrane preparations from control or cardiomyopathic (UM-X7.1) failing hamster hearts. The assay system was the same as in Methods except that the concentration of MgATP was varied as indicated. Each value is a mean ± S.E. of 7 - 10 experiments.

TABLE XX

ENZYME ACTIVITIES OF HEART MEMBRANES PREPARED FROM FAILING HAMSTER HEART HOMOGENATES IN THE ABSENCE OR PRESENCE OF DIFFERENT SUBCELLULAR FRACTIONS OF THE FAILING HEARTS

The assay systems were the same as in Methods. Results for adenylate cyclase activity were expressed as pmoles cyclic AMP formed/mg protein per min. Results for Mg^{++}ATPase, Ca^{++}ATPase and Na^{+}- K^{+}ATPase were expressed as μmoles Pi/mg protein per h. Mitochondria and heavy microsomes isolated from failing hearts were added to the homogenates of failing hearts and the membrane fraction was isolated as described in Methods. Failing hearts were obtained from cardiomyopathic hamsters (UM-X7.1) at advanced degree of congestive heart failure. Each value is a mean ± S.E. of 4 experiments.

Fraction added	Adenylate cyclase	Ca^{++} ATPase	Mg^{++} ATPase	Na^{+}- K^{+} ATPase
-	237 ± 19	31.2 ± 1.8	22.5 ± 1.3	3.4 ± 0.6
Mitochondria	224 ± 15	30.4 ± 1.4	21.0 ± 1.6	3.1 ± 0.5
Heavy microsomes	232 ± 21	30.7 ± 1.6	22.5 ± 1.1	3.3 ± 0.4

DISCUSSION

We have isolated heart sarcolemmal fraction which consists of empty membrane sacs and contains active Na^{+} - K^{+}ATPase, Mg^{++}ATPase, Ca^{++}ATPase and adenylate cyclase. Electron microscopic examination and marker enzyme studies revealed little or no contamination by nuclei, myofibrils, mitochondria, lysosomes or sarcoplasmic reticulum. Unlike mitochondria and sarcoplasmic reticulum, the heart sarcolemmal fraction did not show any ATP supported Ca^{++} binding. Several other investigators[19, 144, 145] have also failed to observe ATP dependent Ca^{++} binding to the skeletal muscle membranes. On the other hand, Severson et al.[80] have recently reported that the skeletal muscle sarcolemma possessed the ability to bind calcium in an ATP dependent manner. It is possible that we may have damaged the energy linked calcium binding mechanism in our preparation during the isolation procedure. It is not our intention to claim that the membrane fraction obtained in this study is completely pure, particularly when our preliminary experiments with sucrose density gradient have revealed the presence of 10 - 15% of inert proteins. It was, however, noted that the specific activities of the membrane bound enzymes did not differ in sarcolemmal preparations obtained from heart homogenates in the absence or presence of exogenously added subcellular fractions. In view of the reproducibility, yield and the speed of isolation, we feel that this preparation offers considerable promise for biochemical, pharmacological and pathological investigations.

The properties of Na^{+} - K^{+}ATPase, a membrane bound enzyme, have been studied in different tissues[17, 18]. Various investigators[19, 59, 71 - 81] have reported the presence of Na^{+} - K^{+}ATPase, Mg^{++}ATPase, Ca^{++}ATPase and

adenylate cyclase in heart and skeletal muscle preparations obtained by different procedures; however, no report demonstrating the activities of all these enzymes in the same heart preparation has yet appeared in the literature. It should be noted that the specific activity of $Na^+ - K^+$ ATPase in our preparation was about 10 μmoles Pi/mg per h, whereas that reported by other investigators[59, 71, 76] for heart sarcolemma varied from 5 - 8 μmoles Pi/mg per h. Although Kidwai et al.[75] have reported the specific activity of $Na^+ - K^+$ ATPase in heart membranes to be about 18 μmoles Pi/mg per h, their results are of preliminary nature and their preparation is not sufficiently characterized for critical evaluation. Furthermore, the specific activity of adenylate cyclase in our preparation was about 280 pmoles cyclic AMP/mg per min, whereas that observed by other investigators[76] for heart sarcolemma was 246 pmoles/mg per min. On the basis of our results on $Na^+ - K^+$ ATPase and adenylate cyclase activities, we have observed 7 - 8 fold enrichment and about 90% recovery of these enzymes in the membrane fraction. Both hypotonic shock and mild LiBr treatment have led to the success of our method. Hypotonic shock aided considerably in extracting cytoplasmic particulate contaminants whereas LiBr treatment removed as much as 60% of the proteins without inactivating the membrane bound enzymes. The method employed in this study is a modification of that of Kono and Colowick[135] and is essentially similar to that reported recently by Severson et al.[80] for the skeletal muscle sarcolemma.

Extraction of the membrane fraction with high concentration (2 M) of NaI or LiBr increased the $Na^+ - K^+$ ATPase activity by about 50%. The specific

activities of $Na^+ - K^+$ATPase in the membrane preparation with or without high salt concentration treatment were markedly lower than that of the solubilized enzyme preparation obtained after deoxycholate and NaI treatments. The low activity of this enzyme in the membrane fraction may reflect more the "native state" of these membranes because $Na^+ - K^+$ATPase is a latent enzyme which is activated by a variety of structure disrupting agents[17]. The observed increase in the enzyme activity after exposure of the membranes to high salt concentrations of NaI or LiBr may be due to some conformational changes in the membranes. At any rate, it was interesting to observe that the $Na^+ - K^+$ATPase of the fraction with or without NaI treatment showed similarities in many kinetic properties, such as concentrations of Na^+ or K^+ for 50% of the maximal activities, and concentrations of ouabain or calcium for 50% inhibition, with some minor differences. Likewise, the pH optimum and the ability of different monovalent cations to substitute for K^+ were similar for all the three preparations. Although the value of K_m for the solubilized $Na^+ - K^+$ATPase preparation was lower than that for the membrane fraction, this should not be interpreted that the enzymes in these preparations are different from each other. In this regard it should be pointed out that since the preparations employed in this study are vesicular in shape, the diffusion of substrate may have an important role in the activity of the enzyme. Thus, different shapes of the vesicles may alter the accessibility of substrate to the catalytic sites without changing the properties of the enzyme per se.

From the apparent sigmoidal shape of the curves for substrate velocity

reactions using varying amounts of Na^+, K^+ and MgATP in the incubation medium, the heart $Na^+ - K^+$ATPase can be considered to exhibit an allosteric nature of activation kinetics. This is further supported by the fact that n values obtained from Hill plots of the data were greater than one. These results indicate co-operativity of interaction between the activating ions as well as of the substrate. The allosteric nature of this enzyme is also supported from our data[146] with conformational changes of $Na^+ - K^+$ATPase during its activation by Na^+ and K^+. The interaction of this enzyme with ouabain, a specific inhibitor, under different experimental conditions has also been suggested to provide evidence for an allosteric site[113, 114]. It is perhaps this allosteric transition which accounts for the ability of this enzyme to adapt to varying conditions in its role as a "$Na^+ - K^+$ pump" at the level of the cell membrane.

The inhibition of $Na^+ - K^+$ATPase by ouabain has been suggested to be the molecular mechanism of the positive inotropic action of this agent[18]. Although Ca^{++} has been also found to have an inhibitory effect on this enzyme system, the contribution of this effect towards positive inotropism due to calcium can not be stated with certainty. This is because of the fact that calcium is known to possess a direct action on the contractile unit[147]. Although some investigators[148, 149] have shown that $Na^+ - K^+$ATPase is inhibited by catecholamines and tyramine, we have failed to show any effect on this enzyme of various sympathomimetic agents or cyclic AMP which are known to have inotropic effects[20, 21, 150]. This discrepancy in results may be due to differences in the source of $Na^+ - K^+$ATPase since these workers[148, 149] have employed microsomal preparations in their studies. At any rate, we do not consider that

the inhibition of sarcolemmal Na^{+} - K^{+}ATPase is a universal requirement for the augmentation of myocardial contractility.

In this study we have demonstrated in the heart membrane fraction the presence of an active ATPase complex that is stimulated by Ca^{++} or Mg^{++}. The Ca^{++}ATPase and Mg^{++}ATPase activities in this fraction cannot be attributed to the presence of myosin or myofibrillar contaminants, since extraction with LiBr is believed to remove most of these proteins. Furthermore, the lack of influence of K^{+} on the Ca^{++}ATPase of this fraction also supports this view. The presence of Ca^{++}ATPase and Mg^{++}ATPase in the heart and skeletal muscle sarcolemma has also been reported in the literature[19, 59, 74, 77]. It should be noted that unlike the sarcoplasmic reticular Ca^{++}ATPase[151], the sarcolemmal Ca^{++}ATPase does not require Mg^{++} for ATP hydrolysis. The specific activities of Ca^{++}ATPase and Mg^{++}ATPase were similar and both were inhibited by an excess of ATP in the incubation medium. Although the K_m value for Ca^{++}ATPase was greater than that for Mg^{++}ATPase, the data at hand do not give the required information to state that Ca^{++}ATPase is a different enzyme from Mg^{++}ATPase. In this regard it should be pointed out that this enzyme complex has been found to be stimulated by other divalent cations such as Mn^{++}, Co^{++} and Ni^{++}. On the other hand, the possibility that Mg^{++}ATPase and Ca^{++}ATPase may be two different components of the sarcolemma cannot be ruled out at present. Ca^{++} ATPase in the membrane has been considered to play a role in Ca^{++} efflux[19, 59] and this enzyme system can be conceived to serve as a "Ca^{++} pump" at the level of the cell membrane. However, it was interesting to find out that

neither Mg^{++}ATPase nor Ca^{++}ATPase of the membrane preparation were influenced by sympathomimetic agents or cyclic AMP and, thus, cannot be regarded as a possible mechanism for the positive inotropic effects of these interventions.

Ca^{++}ATPase was found to be depressed by Na^{+}. This may be due to the competition for membrane sites between Ca^{++} and Na^{+}[22]. The ATP hydrolysis due to 8 mM Ca^{++} was also inhibited by various divalent cations; the order of potency being $Ni^{++} > Co^{++} > Mg^{++} > Mn^{++}$. However, the ATP hydrolysis in the presence of 4 mM Ca^{++} was inhibited only by Co^{++} and Ni^{++}. Since all these divalent cations have been shown to stimulate ATP hydrolysis in this membrane fraction, their inhibitory effects on ATP hydrolysis seem to be complex in nature. We do not have any information at present on the type of inhibition exerted by these divalent cations, however, these are likely to compete for the same sites. Selective inhibition of the transmembrane Ca^{++} conductivity of mammalian myocardial fibers by Ni^{++}, Co^{++} and Mn^{++} has also been reported[67, 69, 152]. In the light of these observations and the data at hand, it is tempting to speculate that Ca^{++}ATPase may also be involved in some manner in the inward directed Ca^{++} currents, thus playing a role in the process of excitation-contraction coupling in the cardiac cell.

The presence of adenylate cyclase activity in heart membranes and its activation by catecholamines or NaF is in agreement with earlier reports on plasma membranes from guinea pig hearts[76] and rabbit skeletal muscle[80]. The K_m for ATP of this enzyme found in the membrane preparations was about 0.08 mM, which is identical to that reported by Drummond and Duncan[86] for the heart washed

cell particles. The broad pH optimum and the Mg^{++} optimum for this enzyme are essentially similar to earlier reports in which the properties of adenylate cyclase were extensively studied using heart washed cell particles and sarcotubular membranes[85 - 87]. Both NaF and epinephrine increased the velocity of the reaction at all Mg^{++} concentrations without any effect on the K_m value for the substrate. These results support the conclusion of Drummond et al.[87] that the stimulatory effects of these agents are due to an increase in the reactivity of the catalytic site with substrate.

Our results concerning the dose response with epinephrine and NaF showed that the adenylate cyclase in the heart membranes was stimulated by about 35 and 400% on addition of epinephrine and NaF respectively. Although the extent of activation of adenylate cyclase by epinephrine was similar to that reported by Tada et al.[76] the activation due to NaF was 8 - 10 times greater than that found by these workers; this appears to be a species difference in addition to differences in methods of isolation of these membranes. At any rate, the activation of adenylate cyclase by directly acting catecholamines, such as epinephrine, norepinephrine and isoproterenol, in contrast to the indirectly acting sympathomimetic agents[150] such as tyramine, ephedrine, metaraminol and nicotine, suggests the presence of β-adrenergic receptors in the heart membrane preparation. This is further supported by our observation that a well known β-adrenergic blocking agent, propranolol, blocked adenylate cyclase activation due to epinephrine. Such findings point the way to further investigation for studies on drug interaction employing the membrane preparation isolated according to

the methods employed in this study.

Although various cations such as Ca^{++}, Mg^{++}, Na^{+} and K^{+} are known to have profound effects on myocardial contractility and metabolism[22 - 26, 147], their exact role in the maintenance of membrane function is not clear. In the present study we have observed that the activities of the membrane bound enzymes such as Mg^{++}ATPase, Ca^{++}ATPase, Na^{+} - K^{+}ATPase and adenylate cyclase, were not changed upon perfusing the isolated rat heart with Mg^{++} free medium, under which conditions the contractility of the myocardium was also not altered. On the other hand, perfusion of the hearts with Na^{+} free or K^{+} free medium resulted in a depression of the Na^{+} - K^{+}ATPase activity under which conditions the hearts were unable to generate contractility. Since the activity of Na^{+} - K^{+}ATPase is regulated by the intracellular concentration of Na^{+} and extracellular concentration of K^{+} [17, 18], the observed reduction in Na^{+} - K^{+} ATPase activity under Na^{+} free or K^{+} free perfused conditions is not surprising. It should be noted that perfusion of the hearts with Na^{+} free medium can be conceived to result in a marked reduction in the intracellular Na^{+}. The decrease in Na^{+} - K^{+}ATPase activity seems unlikely to be due to changes in membrane integrity since the activities of Ca^{++}ATPase, Mg^{++}ATPase and adenylate cyclase were not altered under these conditions. K^{+} deficiency in vivo has also been shown to decrease myocardial Na^{+} - K^{+}ATPase[129]; however, in view of the insufficient information at our disposal, it is very difficult to state with certainty whether or not depression in Na^{+} - K^{+}ATPase activity has anything to do with the cessation of contractile activity in hearts perfused with Na^{+} or K^{+}

free medium.

Not only were the hearts unable to contract upon perfusion with Ca^{++} free medium, but the activities of Na^{+} - K^{+}ATPase, Mg^{++}ATPase and adenylate cyclase were decreased in the membrane preparations. Since the loss of contractility upon Ca^{++} free perfusion has been shown to occur within 30 s[153] and the changes in membrane bound enzyme activities were not significant until 10 min of Ca^{++} free perfusion, it is unlikely that the observed alterations in enzyme activities contribute towards the impairment of contractility. On the other hand, these changes are likely to be due to damage to the membrane integrity because 10 min of perfusion of the heart with Ca^{++} free medium has been shown to produce separation of intercalated discs and separation of the basement membrane from the plasma membrane[153]. Other workers[154, 155] have also shown marked ultrastructural changes in the myocardium upon perfusion with Ca^{++} free medium.

It was interesting to note that the responses of adenylate cyclase to NaF in the membrane fraction from hearts perfused with Ca^{++} free medium were augmented. This may be due to the removal of some inhibitor from the cell membrane in addition to the changes in the integrity of the plasma membrane upon perfusion with Ca^{++} free medium. Although Ca^{++}ATPase activity did not change on perfusing the heart with Ca^{++} free medium, it is possible that this enzyme is more resistant to the membrane changes induced under this condition. A decrease in Ca^{++}ATPase in addition to marked changes in the activities of Mg^{++}ATPase, Na^{+} - K^{+}ATPase and adenylate cyclase were apparent in the membrane fraction

isolated from hearts which had undergone successive perfusion with Ca^{++} free followed by Ca^{++} containing media. Extensive damage to myocardial ultrastructure has been reported to occur under this calcium paradoxical condition[155, 156]. Thus the degree of membrane damage may determine the extent of changes in membrane bound enzyme activities. Although the exact mechanisms behind these membrane changes in hearts perfused with Ca^{++} free medium is not clear at present, the results from this study suggest the role of extracellular Ca^{++} in the maintenance of membrane bound enzyme activities.

In cardiomyopathic hamsters (UM-X7.1) with an advanced degree of heart failure, the activities of Mg^{++}ATPase, Ca^{++}ATPase and Na^{+} - K^{+}ATPase were decreased. Such a decrease is unlikely to be due to contaminating mitochondrial or microsomal proteins since no difference in the activities of membrane bound enzymes was observed when the sarcolemmal fractions were isolated from failing heart homogenates in the absence or presence of exogenously added mitochondria or heavy microsomes. Although the basal adenylate cyclase activity of the membrane fraction obtained from failing hearts was not different from the control, the responses of this enzyme to NaF and epinephrine were markedly depressed. These changes in the membrane bound enzyme activities can be interpreted to reflect extensive damage to the sarcolemma in failing hearts. Such a change in membrane integrity may be due to alterations in the lipoprotein composition of the plasma membrane. Further experiments are, therefore, needed to study the chemical composition of the failing heart sarcolemma in order to gain broader appreciation of the underlying mechanisms behind alterations in membrane

bound enzyme activities.

Since cardiomyopathic hamsters with an advanced degree of heart failure were employed in the present study, it is very difficult to comment upon the significance of the observed changes in membrane bound enzyme activities. Previous studies[28, 29, 93, 128, 131, 132, 157] from this laboratory on adenylate cyclase of washed cell particles and $Na^+ - K^+$ ATPase solubilized preparation in different types of failing hearts, have suggested an association of membrane abnormality with heart failure. The present study dealing with membrane bound enzyme activities in a new strain of cardiomyopathic hamsters strengthens this belief. Other investigators[27, 89, 91, 92, 98, 127, 130] have also reported dramatic alterations in the activities of adenylate cyclase in the absence and presence of NaF or hormones and $Na^+ - K^+$ ATPase in different experimental models of heart failure. Further studies concerning changes in membrane bound enzyme activities at earlier stages of heart failure are needed in order to draw conclusions regarding membrane abnormalities in the pathogenesis of this disease.

CONCLUSIONS

In addition to isolating heart sarcolemma containing Na^+ - K^+ATPase, Mg^{++}ATPase, Ca^{++}ATPase and adenylate cyclase activities, the properties of the dog heart membrane bound enzymes were examined in this study. Furthermore, the role of various cations such as Ca^{++}, Mg^{++}, Na^+ and K^+ in the membrane integrity was examined by monitoring the activities of the enzymes in the membranes obtained from the rat hearts perfused with medium in the absence of these cations. The possibility of membrane abnormality in heart failure was also tested by determining the membrane bound enzyme activities in hearts of cardiomyopathic hamsters (UM-X7.1) with an advanced degree of heart failure. The following conclusions are drawn:

1. Hypotonic shock and LiBr treatments of the heart homogenates yielded membrane preparation with substantial Na^+ - K^+ATPase, Mg^{++}ATPase, Ca^{++}ATPase and adenylate cyclase activities which hopefully will be of use for biochemical and pharmacological studies.

2. Na^+ - K^+ATPase in the heart membranes was sensitive to ouabain and calcium and exhibited allosteric properties.

3. The Ca^{++}ATPase in the heart membranes was inhibited by various ions such as Mg^{++}, Mn^{++}, Ni^{++}, Co^{++} and Na^+ and thus may be considered to be implicated in the process of excitation-contraction coupling at the level of cell membrane.

4. The heart membranes contained hormone sensitive adenylate cyclase with intact β -adrenergic receptor mechanisms.

5. Various sympathomimetic agents and cyclic AMP, which increase myocardial contractility, did not influence the activities of Mg^{++}ATPase, Ca^{++}ATPase and Na^{+} - K^{+}ATPase.

6. Extracellular calcium seems to play an important role in maintaining the activities of membrane bound enzymes such as Na^{+} - K^{+}ATPase, Mg^{++}ATPase and adenylate cyclase.

7. Alteration in the enzyme activities of the membrane fraction from the failing hearts provided further support to the possibility of membrane abnormality in heart failure.

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