SOLUBILIZATION, PURIFICATION AND CHARACTERIZATION OF A DIVALENT CATION DEPENDENT ATPase FROM RAT HEART SARCOLEMMA

A Thesis

Presented to the

University of Manitoba

In Partial Fulfilment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

BALWANT SINGH TUANA

Department of Physiology

Faculty of Medicine

July 1982

SOLUBILIZATION, PURIFICATION AND CHARACTERIZATION OF A DIVALENT CATION DEPENDENT ATPase FROM A RAT HEART SARCOLEMMA

BY

BALWANT SINGH TUANA

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

DOCTOR OF PHILOSOPHY

ACKNOWLEDGEMENTS

It is indeed my pleasure to acknowledge the excellent guidance of my supervisor and friend Dr. Naranjan S. Dhalla during the course of my studies. His valuable suggestions, constructive criticism and constant encouragement will always be appreciated. I would also like to thank Drs. N.L. Stephens, E. Kroeger, M.S. Nijjar and H.G. Friesen for their constructive advice. I am much indebted to the staff of the Physiology Department for their kindness and consideration during the course of my studies.

I would like to extend my appreciation to Dr. P.K. Singal, V. Panagia, S. Takeo, J.M. Lamers, P.K. Das, M. Karmazyn and A. Dzurba for their cooperation and moral support. I would also like to thank Mike Daly, Clayton Heyliger, Grant Pierce, Mike Kutyric, Gina Taam, Marnie Moffat, Nina Kapoor, Lauri Alto, Raj Bhullar, Pat Gillevet, John Rowe and Vergil Nathanial for all their help and friendship.

I would like to thank Darlene Simmons, and Lauri and Glenn for their help in the preparation of this manuscript.

I am highly indebted to the Canadian Heart Foundation for providing me with the financial support during the course of this work.

The work in this thesis was supported by grants to Dr. N.S. Dhalla from the Medical Research Council.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SOLUBILIZATION, PURIFICATION AND CHARACTERIZATION OF A DIVALENT CATION DEPENDENT ATPase FROM RAT HEART SARCOLEMMA

ABSTRACT

Heart sarcolemma exhibits an ATPase activity that is dependent on divalent cations such as Ca^{2+} or Mg^{2+}. Trypsin treatment of sarcolemma resulted in the solubilization of a Ca^{2+} - specific ATPase activity, with retention of the divalent cation dependent ATPase activity in the 100,000 x g pellet. This divalent cation dependent ATPase was solubilized by sonication of the pellet with 1% Triton X-100. In an attempt to purify the dog heart sarcolemmal divalent cation ATPase the solubilized enzyme was subjected to column chromatography on a Sepharose-6B column, followed by ion-exchange chromatography on a DEAE cellulose column. The enzyme preparation was found to be rather labile and did not enter polyacrylamide gels, thus the purity of the sample could not be accurately assessed. The partially purified ATPase preparations did not show any cross-reactivity with dog heart myosin antiserum or with Na^{+} - K^{+} ATPase antiserum. The enzyme was found to be insensitive to the inhibitors, ouabain, verapamil, oligomycin and vanadate. The enzyme preparation did not exhibit any Ca^{2+} stimulated Mg^{2+} dependent ATPase.

The solubilized enzyme from rat heart sarcolemmal membranes was purified by ammonium sulphate fractionation and column chromatography using a hydrophobic column followed by gel filtration on a Sepharose-6B

and Sephadex-G200 column. The purified enzyme was seen as a single protein band in nondenaturing polyacrylamide gel electrophoresis. In sodium dodecyl sulphate acrylamide gels, the enzyme dissociated into three subunits with molecular weights of about 90,000, 80,000 and 67,000. The molecular weight of the enzyme estimated by gel filtration on a Sephadex G-200 column was found to be about 240,000. The ATPase utilized Ca-ATP as a substrate with a K_m of 0.16 mM for the high affinity site and 1 mM for the low affinity site. The ATPase also utilized Mg-ATP as substrate with a K_m of 0.12 mM for the high affinity site and 1 mM for the low affinity site. The enzyme was also able to utilize ITP, CTP, GTP, UTP, ADP and AMP as a substrate but at a lower rate, as compared with ATP. The enzyme was activated by Ca^{2+} with a K_a of 0.4 mM and Mg^{2+} with a K_a of 0.2 mM; it was also activated by other cations in the order $Ca^{2+} > Mg^{2+} > Mn^{2+} > Sr^{2+} > Ba^{2+} > Ni^{2+} > Cu^{2+}$. The ATPase activity in the presence of Ca^{2+} was markedly inhibited by Mg^{2+}, Mn^{2+}, Ni^{2+} and Cu^{2+}. The monovalent cations such as Na^{+} and K^{+} were without effect on the ATPase. The enzyme was insensitive to ouabain, verapamil, D-600, oligomycin, azide and vanadate. Calmodulin failed to stimulate the ATPase and instead showed a slight inhibitory effect. The enzyme did not bind to a calmodulin affinity column. The enzyme was phosphorylated in the presence of the cAMP dependent catalytic subunit of protein kinase, (0.46 mM ^{32}P/mole enzyme) and this was associated with 25 - 30% decrease in activity of the ATPase.

The results demonstrate that a new enzyme, which catalyzes the hydrolysis of ATP in the presence of various divalent cations is present and can be isolated and purified from the heart cell membrane.

I. INTRODUCTION

Heart sarcolemma is intimately involved in the regulation of cardiac contractile activity and is known to contain various enzyme systems which contribute to the function of this membrane. For example, the sarcolemma contains the catecholamine - sensitive adenylate cyclase system, which regulates the activities of a variety of enzymes crucial for heart function. Also present in this membrane, is the $Na^+ + K^+$ ATPase, which is involved in pumping Na^+ out of the cardiac cell. In addition, the sarcolemma has been shown to contain a Ca^{2+} stimulated Mg^{2+} dependent ATPase which is thought to be involved in "Ca^{2+}" efflux from the heart cell. In this regard the Ca^{2+} "pump" utilizes Mg - ATP as a substrate and is activated by micromolar concentrations of Ca^{2+}. On the other hand, cytochemical studies on the cardiac cell indicate the presence of a divalent cation ATPase activity that is dependent on Ca^{2+} or Mg^{2+} at the sarcolemmal membrane. The divalent cation dependent ATPase has also been demonstrated to be present in isolated sarcolemmal membrane preparations by various investigators. This ATPase is activated by millimolar concentrations of divalent cations such as Ca^{2+} or Mg^{2+} and has been demonstrated to be different from the Ca^{2+} stimulated Mg^{2+} dependent ATPase of the sarcolemma. Trypsin treatment of heart sarcolemma was observed to result in the solubilization of a Ca^{2+} dependent ATPase activity that was not activated by Mg^{2+}. However, there still remained, in the trypsin treated sarcolemmal pellet, a

divalent cation dependent ATPase activity. This divalent cation dependent ATPase has been implicated in opening Ca^{2+} "channels" to allow the passive entry of Ca^{2+} into the myocardial cell. At present, the biochemical nature and the physiological role of the cation dependent ATPase still remains to be established. It is the purpose of this study to isolate, purify and characterize the divalent cation dependent ATPase of the sarcolemma so that its structure and role in heart cell function can be determined.

II. REVIEW OF LITERATURE

It is generally believed that the various membrane systems such as the sarcoplasmic reticulum, mitochondria and sarcolemma play a central role in heart function and metabolism (1). The main role of sarcoplasmic reticulum in heart cell function is thought to be the regulation of cytosolic calcium ion concentration. During depolarization of the cell, calcium is released from the sarcoplasmic reticulum, and this results in increased cytosolic calcium concentration and activation of the myofilaments, leading to cardiac contraction. Relaxation of the myocardium is brought about by lowering the cytosolic calcium concentration, which is achieved, in part, by the calcium pump mechanism of the sarcoplasmic reticulum. The calcium pump has been identified as a protein of Mr = 100,000, and is referred to as the calcium stimulated magnesium dependent ATPase (Ca^{2+} + Mg^{2+} ATPase). The (Ca^{2+} + Mg^{2+}) ATPase is stimulated by micromolar concentration of calcium and utilizes Mg - ATP as a substrate. The substrate, Mg - ATP, binds to the cytoplasmic surface of the enzyme together with Ca^{2+}; ATP hydrolysis results in the release of ADP, formation of E $\sim$ P and simultaneous translocation of calcium into the luminal surface of the sarcoplasmic reticulum, where it is stored as a complex with calsequestrin; a protein of Mr = 60,000. The (Ca^{2+} + Mg^{2+}) ATPase is regulated by both cyclic AMP - protein kinase (cAMP - Pk) and calmodulin dependent phosphorylation. The phosphorylation effect is mediated through a protein of Mr = 22,000; known as phospho-

lamban. Although very little is known about the calcium release mechanism of the sarcoplasmic reticulum, recent evidence indicates that a group of intrinsic proteins of the membrane may be involved (for review see - 2, 3).

The main role of mitochondrial membrane in heart function is the synthesis of ATP. This is achieved through membrane bound proteins, whose structure and arrangement is of fundamental importance to carry out the oxidation - reduction reactions, which give rise to the electron-motive force. The energy of the electron - motive force, in the form of membrane potential and a pH gradient is transduced into chemical energy in the form of ATP by the mitochondrial ATPase system. The mitochondrial ATPase is a protein of Mr = 347,000 and consists of several subunits (review, see - 4,5). Although mitochondria are also capable of accumulating and releasing calcium under a wide variety of in vitro conditions, their involvement in the cardiac contraction - relaxation cycle is not fully appreciated. It is generally held that mitochondria serve as a calcium sink under pathological situations but the mechanism for their calcium transporting property is far from clearly understood.

The sarcolemmal membrane not only constitutes the real permeability barrier of the cell and gives rise to its electrical characteristics, but is also involved in the functions such as ion and substrate transport, communication and secretion (6, 7). The various functions performed by the sarcolemma are determined by the architecture of the

various molecular components of the membrane. The term sarcolemma as used here includes the lipid bilayer, the glycoprotein coat located on the immediate outer surface of the lipid bilayer and an integral part of the membrane, the external lamina or carbohydrate coating just superficial to the cell coat. Due to the complexity of the sarcolemma, only recently procedures have become available to obtain purified membrane preparations so that the various components such as carbohydrates, lipids and proteins can be studied. Since this investigation is concerned with some enzyme functions of the sarcolemmal membrane the following section is devoted for providing detailed information on different aspects of this membrane system.

A. Sarcolemmal Stucture and Function.

In electron microscopic studies on intact cells, the sarcolemmal surface appears as a uniformally thick ($\simeq$ 50 nm) mat or network of fine filaments (8). In tissue preparations the surface appears layered with a less dense inner zone ($\simeq$ 20 nm) known as the surface coat, and a slightly denser outer zone ($\simeq$ 30 nm) referred to as the external lamina (9). Whether this layering represents a true structural and chemical difference is not known. In a study of cell coats (10), it was pointed out that the glycoprotein chains of the surface coat probably extend out from the lipoprotein layer about 20 - 30 nm but that, under conditions of tissue preservation, the filaments may collapse and form the mat-like network seen in the electron microscope.

Histochemistry of the myocardial cell surface has identified the presence of sialic acid and other acidic carbohydrates (11). The use of colloidal iron stain indicated the presence of negatively charged sialic acid residues at the surface coat next to the lipid bilayer and on the outer-most region of the external lamina (12). These negatively charged sialic acid molecules have been implicated in calcium binding and considered to be the sites in the membrane from which calcium that enters the cell during depolarization is derived (13). But just how important this part of the membrane is in cell function and in regulating ionic binding is as yet unclear. In a number of studies on the isolated sarcolemmal membrane, the presence of both high and low affinity calcium binding sites has been demonstrated (14, 15). It was postulated that the low affinity calcium binding sites may represent specific sites for calcium entry, at the sarcolemmal surface, since the apparent association constant of these sites correlated with the association constant for calcium required to increase contractility in intact hearts (16).

According to the model of Singer and Nicolson (17) a biological membrane consists of a lipid bilayer, with protein molecules specially arranged according to their structure and function. The hydrophillic components of the various molecules, whether lipids or proteins, are exposed to the extracellular or the cytosolic surface, whereas the hydrophobic components are embedded in the core of the membrane. The whole system is in a constant dynamic motion, with the lipid molecules undergoing lateral and perhaps "flip-flop" movements. The protein

molecules also undergo lateral mobility in the plane of the membrane, although at a slower rate as compared with that for the lipids (18). The chemical composition of membranes varies from one to another, and is related to the function of that particular membrane system, cell or organ. Thus in the myelin membranes sheathing the nerve fibres, there is about ten times more lipid present in comparison to protein, while the mitochondrial membrane contains equimolar amounts of lipid and protein. The function of the myelin membrane is primarily as an insulator, excluding substances that interfere with transmission of nerve impulses. Mitochondria[1] membrane, on the other hand, serves the function of organizing in space and perhaps in time, the action of many enzymes associated with the mitochondria; the high protein content of the membrane is due to these enzymes. Most membranes are known to contain a number of different proteins, which considering the variety of metabolic jobs performed by most membrane types, is not surprising. Membrane proteins in general are characterized by a relatively high proportion of amino acid residues with long non-polar side chains such as leucine and isoleucine. These are thought to associate proteins with lipids through their affinity for the fatty acid chains present in the lipids. However, very few membrane proteins have been fully characterized in terms of their chemical composition and molecular structure, primarily due to the difficulty of extracting intact proteins from their associated lipids.

B. Some Sarcolemmal Proteins, and their Functions.

(i). Sodium and potassium-activated adenosine triphosphatase.

The $Na^+ - K^+$ ATPase discovered by Skou (19) has been shown to transport Na^+ and K^+ across the cell membrane. This enzyme is ubiquitous among animal species and in all animal cells but its activity varies over a wide range. Highest $Na^+ - K^+$ ATPase activities have been noted in excitatory and secretory tissues: brain cortex, kidney outer medulla and heart (20, 21). The presence of $Na^+ - K^+$ ATPase in the cell is usually restricted to the plasma membrane, and the enzyme is considered as a marker enzyme for the plasma membrane. This enzyme utilizes ATP as a substrate, which is hydrolyzed on the cytosolic face of the membrane, and 3 Na^+/ATP molecule are transported to the extracellular fluid. In erythrocytes (22) and squid giant axon (23) this occurs in exchange for 2 K^+, thus building up an electrochemical gradient.. Reconstitution studies with the purified enzyme, incorporated into phosphatidylcholine vesicles has confirmed the electrogenic 3 Na^+/2 K^+ stoichiometry (23). The Na^+ - pump mechanism explains why under normal steady-state conditions the cytoplasm of most cells, in contrast to the extracellular fluid, has a high K^+ concentration but a relatively low Na^+ concentration.

The $Na^+ - K^+$ ATPase has been purified from the mammalian kidney (24), shark rectal gland (25) eel electroplax (26), and duck salt gland (27), and partially purified (to about 50% purity) from tissue sources, such as beef brain (28) and beef heart (29). Two essentially different methods have been utilized successfully to purify the enzyme.

The first method involves the use of high detergent concentrations to solubilize the enzyme from the membrane (30). The solubilized $Na^+ - K^+$ ATPase extract is further purified by molecular - sieve chromatography (31) ammonium sulfate fractionation (30) and sucrose density-gradient centrifugation (25). In the second method, $Na^+ - K^+$ ATPase is not solubilized from the membrane, but contaminating proteins are solubilized at low detergent concentrations, followed by sucrose density-gradient centrifugation yielding a pure preparation (24).

Studies on the purified $Na^+ - K^+$ ATPase indicate that the enzyme has a molecular weight of about 274,000 to 280,000, regardless of species (32). The holoenzyme is a tetramer of two large subunits, Mr = 100,000 and two small subunits Mr = 40,000. Phosphorylation by ATP occurs in the subunit with Mr = 100,000. This subunit also contains the binding site for ATP (33) and the specific sites for inhibition by the sulfhydryl reagent N-ethyl maleimide and the cardiac glycoside ouabain (34, 35). The coincidence of the two 100,000 subunits of the $Na^+ - K^+$ ATPase in gel electrophoresis as one band does not imply that they are chemically identical and that they would both have a phosphorylation site. Only one site per 280,000 molecular weight complex is phosphorylated by ATP, just as there is also only one binding site for ATP and ouabain (36, 37). The subunit with Mr = 40,000 has been identified as a sialoglycoprotein (37). In lipid bilayers, the glycoprotein shows ionophoric properties for Na^+, NH_4^+,

Cs^+, Rb^+, and Li^+ but not for K^+. However, in the presence of the 100,000 subunit, the selectivity for Na^+ was increased and optimal Na^+ ionophoric activity was obtained at a subunit ratio of one 100,000 to two 40,000 subunits (38). The subunit with Mr = 40,000 is also involved in the overall $Na^+ - K^+$ ATPase reaction, since this reaction is inhibited by the antibody against this subunit (39), and by plant lectins which selectively bind to specific carbohydrates in this glycoprotein (40). In addition, the 40,000 subunit co-purifies with the 100,000 subunit (32), indicating there is an essential interaction between the subunits.

Recently, the presence of a third subunit of Mr = 12,000 has been reported by some investigators (41, 42). This small subunit was not observed in earlier studies with highly purified $Na^+ - K^+$ ATPase preparations because the acrylamide concentrations in the SDS-polyacrylamide gel electrophoresis used to examine the protein subunits were too low to separate this subunit from the tracking dye front (41). Forbush et al. (43) observed that the small subunit is a proteolipid i.e. a hydrophobic protein and is soluble in organic solvents such as chloroform: methanol mixtures (44). Reeves et al. (41) isolated two proteolipid components of Mr = 12,000 from highly purified lamb kidney $Na^+ - K^+$ ATPase by chromatography of the enzyme on Sepharose CL-6B, followed by extraction with a chloroform: methanol solvent and chromatography on Sephadex LH-60. It has been suggested that the proteolipid forms an ion channel through the membrane (45).

The proteolipid, which is also associated with cardiac $Na^+ - K^+$ ATPase, has been shown to be a site of ouabain binding (46); it may be that the inhibition of $Na^+ - K^+$ ATPase by cardiac glycosides is due to blocking of a channel. Dowd et al. (47) demonstrated that the proteolipid is also a site of phosphorylation, and speculated that this protein may be involved in regulating the activity of cardiac $Na^+ - K^+$ ATPase.

The $Na^+ - K^+$ ATPase, like most membrane bound enzymes, requires phospholipids for activity (48). Lipid removal from the enzyme by detergents, organic solvents or phospholipase treatment (49) leads to partial or complete inactivation of the enzyme. The major phospholipids associated with the purified $Na^+ - K^+$ ATPase are phosphotidylcholine, phosphotidylethanolamine, phosphotidylserine and phosphotidylinositol. In the purified preparation there are about 270 mols phospholipids per mole of enzyme, some of which are acidic phospholipids (phosphotidylserine and phosphotidylinositol). Delipidation of the $Na^+ - K^+$ ATPase preparations leads to inactivation of the enzyme, which can be maximally reactivated by the addition of phosphotidylserine and phosphotidylinositol. This led some investigators to conclude that the transport system consists of a complex of the $Na^+ - K^+$ ATPase protein and phosphotidylserine. However, recent evidence indicates that after full replacement of endogenous phospholipids by phosphotidylcholine, the substituted enzyme when incorporated in phosphotidylcholine vesicles exhibits sodium transport

and only a partial loss of ATPase activity (50). Thus it appears that the interactions between the various subunits of the Na^+ - K^+ ATPase molecule and its surrounding phospholipids regulate the activity of the enzyme and the sodium pump.

(ii). Adenylate Cyclase System.

Adenosine-3'-5'-monophosphate (cAMP) is now recognized as an ubiquitous secondary messenger molecule, controlling diverse metabolic processes. In the heart cell, cAMP is involved in the regulation of metabolism and cardiac contractility by controlling the activities of various enzymes (51) as well as by influencing Ca^{2+} movements across various membrane systems (52 - 54). The synthesis of cAMP in cells is catalysed by the hormone-sensitive adenylate cyclase system, which is mainly associated with the plasma membrane. Plasma membrane preparations from a wide variety of tissues display adenylate cyclase activity, which can be activated by the addition of appropriate hormones or analogues to the assay mixture. However, the extent of hormonal activation assayed in vitro is generally less than that observed when the synthesis of cAMP is studied in the intact cell or tissues. Hormonal stimulation of adenylate cyclase requires the presence of guanine (or related purine) nucleotides in addition to substrate. It is found that GTP and its analogues can stimulate adenylate cyclase activity in the absence of hormones. Fluoride is another stimulatory ligand of eukaryotic adenylate cyclase, however the physiological significance of this effect is unclear. The catecholamine-sensitive adenylate cyclase system is

composed of at least three distinct components: the β-adrenergic receptor, the adenylate cyclase enzyme and a transmitter molecule that binds guanine nucleotides.

It is now clear that receptors for hormones are indeed individual proteins distinct from adenylate cyclase. Direct experimental evidence for this has come from genetic manipulation to resolve receptor and enzyme. Insel et al. (55) demonstrated that there are two clones of cultured cells that are phenotypically deficient in adenylate cyclase, but retain β-adrenergic receptors. The β-receptor has been solubilized in an active form by treatment of erthrocyte membranes with glycoside digitonin (56). Other detergents such as lubrol Px, triton X-100 and deoxycholate were found to be ineffective. Using affinity chromatography on immobilized alprenolol linked via a hydrophilic spacer arm to agrose beads, a 12,000-fold purification of the β-receptor has been achieved (57). By SDS-polyacrylamide gel electrophoresis and affinity labelling of the β-adrenergic receptor, a tentative identification of two subunits of Mr = 37,000 and Mr = 41,000 was reported for the β-receptors obtained from rat skeletal myoblasts grown in culture and from turkey erthrocytes (58). Estimation of molecular weight by gel filtration has indicated the value to be in the range of 150,000 - 200,000 (59). Comparison of the molecular weights of the β-receptor obtained by the two different procedures suggest that the receptors are composed of multiple subunits. Orly and Schrawn (60) demonstrated the functional individuality of the β-receptor and the adenylate cyclase. By using Friend

erythroleukemia cells lacking receptor sites and chemically treated turkey erthrocytes with no residual adenylate cyclase activity, these authors showed that a catecholamine-sensitive adenylate cyclase activity could be reconstituted.

Resolution of the β-receptor from the adenylate cyclase enzyme has been recently achieved. Both the receptor and the enzyme can be solubilized from the membrane with the detergent digitonin. These components can be partially separated by gel filtration (61) and completely separated by affinity chromatography (56). The adenylate cyclase enzyme from dog heart sarcolemma was purified 5000-fold using a combination of hydrophobic chromatography and affinity chromatography on ATP-sepharose (62); the molecular weight of the enzyme was estimated to be around 150,000. On the other hand, Neer (63), Haga et al. (64) and Stengel and Hanounce (65) have shown that adenylate cyclase from brain, S4q lymphoma cells and liver are larger proteins (Mr = 2 - 3 x 10^5). However, the adenylate cyclase has not yet been purified to homogenity due to instability of the enzyme, and thus detailed biochemical studies are lacking.

The 5,000-fold purified adenylate cyclase preparation from dog heart sarcolemma, was found to be sensitive of GPP (NH)$_p$ stimulation, suggesting that the nucleotide (GTP) binding component remains attached to the enzyme during this procedure. The GTP-binding proteins from solubilized pigeon erythrocyte membranes were separated from adenylate cyclase by affinity chromatography on a GTP-Sepharose matrix (66). The specifically eluted regulatory protein restored Gpp (NH)p and

NaF-stimulated adenylate cyclase activity to a preparation deprived of the guanine nucleotide-binding proteins. Purification by this procedure (about-60 fold) was improved upon subsequent sucrose density gradient centrifugation (66). Pfeuffer (66) used (^{32}P) GTP γ-azidoanilide to label pigeon erythrocyte membranes, and found that the fractionation of a 42,000 dalton band on dodecyl sulfate-polycrylamide gels was consistent with its involvement with adenylate cyclase. Further support for the involvement of a 42,000-dalton protein in the activation of adenylate cyclase stems from studies with cholra toxin, using (^{32}P) NAD as a substrate for the toxin; both Gill and Meren (67) and Cassel and Pfeuffer (68) were able to label primarily a 42,000-dalton protein. Recent studies indicate that the GTP binding protein has been purified from liver plasma membranes. The purified preparation was found to contain three polypeptides with molecular weights of 52,000, 45,000 and 35,000. The active GTP-binding protein behaves as a multisubunit complex of these polypeptides.

(iii) 5'-Nucleotidase.

The 5'-nucleotidase has been shown to be primarily present in the heart cell membrane and is regarded as a sarcolemmal marker enzyme (69). Experiments with perfused rat heart indicate that the 5'nucleotidase hydrolyzes exogenously added AMP with a concomitant transfer of adenosine across the cell membrane (70). On the basis of these findings, 5'nucleotidase has been implicated as a translocase which converts an intracellular signal in the form of AMP into an

extracellular signal in the form of adenosine (71). Adenosine is a powerful vasodilator, and is known to increase coronary blood flow and the oxygen supply to the heart in an effort to correct the energy deficiency (72).

Purification of the membrane-bound enzyme has been reported from various sources including mouse liver (73), rat cerebellum (74), rat liver (75) and pig lymphocytes (76). Recently, the 5'nucleotidase was solubilized by extraction of rat heart membranes with Triton X-100 (77). Subsequent steps involving affinity chromatography on columns of concanavalin A-Sepharose and ADP-Agarose resulted in an apparent homogenous preparation of the enzyme, as assessed by gel electrophoresis. The specific activity of the purified 5'-nucleotidase from the heart is highest when compared with the activities of the enzyme from other sources. The heart sarcolemmal enzyme is a glycoprotein with a subunit molecular weight of 74,000. The native form of the enzyme is thought to exist as a dimer of molecular weight 147,000. The enzyme hydrolyzes all nucleotide 5'-monophosphates, but with the greatest affinity for AMP. The 5'-nucleotidase is thought to be the main catalyst of adenosine production and thus further work on the purified enzyme from the heart will yield a valuable information with respect to the regulation of adenosine formation, and subsequently coronary blood flow in the heart.

(iv) Calcium Stimulated Magnesium Dependent Adenosine Triphosphatase $(Ca^{2+} + Mg^{2+})$ ATPase.

The $(Ca^{2+} + Mg^{2+})$ ATPase has been described to be present in the

plasma membrane preparations from a wide variety of tissues including heart sarcolemma (78). The (Ca^{2+} + Mg^{2+}) ATPase activity is thought to be a calcium pump mechanism, which is involved in carrying out Ca^{2+} efflux from the cell and thus keeping the internal Ca^{2+} concentration around the micromolar range. The enzyme is fully operative in micromolar concentrations of calcium with a K_a value around 0.1 - 0.5 μM, and utilizes Mg-ATP as a substrate. Recently purified (Ca^{2+} + Mg^{2+}) ATPase from the erythrocyte membrane was found to be a protein of Mr = 145,000, whose activity is intimately regulated by the calcium binding protein, calmodulin (79). The heart sarcolemmal (Ca^{2+} + Mg^{2+}) ATPase was also solubilized by using TX-100, and subsequent chromatography on a calmodulin affinity column yielded a preparation of the enzyme that was about 60% pure. The sarcolemmal enzyme was estimated to have a molecular weight of about 150,000 (80). Calmodulin, an intrinsic component of the sarcolemma, was found to stimulate the (Ca^{2+} + Mg^{2+}) ATPase by increasing the V_{max} and decreasing the K_a value for Ca^{2+} (81). In isolated sarcolemmal vesicles, the (Ca^{2+} + Mg^{2+}) ATPase activity was stimulated by cAMP and Ca^{2+} dependent phosphorylation in a manner similar to that for Ca^{2+} uptake (82). Upon dephosphorylation in the presence of phosphorylase phosphatase, the Ca^{2+} uptake and the (Ca^{2+} + Mg^{2+}) ATPase activities of the vesicles were decreased. These studies provide evidence that the sarcolemmal enzyme is regulated by phosphorylation - dephosphorylation reactions involving cAMP and the Ca^{2+} - calmodulin systems.

Further attempts are being continued to purify the enzyme to homogeneity, and subsequent work will hopefully lead to a better understanding of the mechanism as well as regulation of Ca^{2+} efflux by the sarcolemmal (Ca^{2+} + Mg^{2+}) ATPase.

(v). Calcium Dependent ATPase.

Tuana and Dhalla (83) recently purified a Ca^{2+} - dependent ATPase from rat heart sarcolemma which was not activated by Mg^{2+} ions. Unlike the (Ca^{2+} + Mg^{2+}) ATPase, the sarcolemmal Ca^{2+} - dependent ATPase does not utilize Mg-ATP as a substrate, and is activated by mM concentrations of Ca^{2+} with a K_a value of about 0.15 mM. The very low affinity of the Ca^{2+}-dependent ATPase for Ca^{2+} rule out the role for this enzyme as a possible Ca^{2+} pump mechanism. The Ca^{2+} - dependent ATPase was solubilized from heart sarcolemmal membranes by trypsin digestion (84) and subsequent ammonium sulphate fractionation and column chromatography on Sepharose-6B and DEAE cellulose yielded a purified enzyme preparation. The purified enzyme was found to be a protein of Mr = 67,000, consisting of two subunits of Mr = 55,000 and 12,000. The purified enzyme was not stimulated by calmodulin and was found to be insensitive to the inhibitor vanadate. The enzyme was shown to posses a Na^{+} - Ca^{2+} antagonistic site, and potently inhibited by Mg^{2+} ions. Although the exact role of the Ca^{2+} - dependent ATPase is unclear, it is suggested to be involved in the Na^{+} sensitive Ca^{2+} entry into the myocardial cell, or as a Na^{+} - Ca^{2+} exchange carrier protein. However, further work on the purified enzyme would hopefully clarify the function of the Ca^{2+} - dependent

ATPase in the heart sarcolemmal membrane.

(vi). Divalent Cation Dependent Adenosine Triphosphatase

In addition to (Ca^{2+} + Mg^{2+}) ATPase and Ca^{2+} - dependent ATPase, heart sarcolemma has been shown to exhibit a divalent cation ATPase activity which is dependent on divalent cations such as Ca^{2+}, Mg^{2+} or Mn^{2+} (85). In this regard, a divalent cation dependent ATPase activity has been described in plasma membrane preparations in a wide variety of tissues including muscle (86), liver (87) placenta (88), platelets (89) adrenal medulla (90), mammary gland (91), nerve cells (92), fibroblast (93), erythrocytes (94). In many instances the divalent cation dependent ATPase has been demonstrated to be an ecto-ATPase, i.e. an enzyme whose catalytic site is localized on the extracellular side of the plasma membrane of the cells. Ecto-ATPases utilize exogenous ATP as substrate and their activities are confined to the external surface of the cells (93). Although a unique biological role for ecto-ATPases has not been discovered, their function has been the object of various speculations. It was proposed, for instance that the enzyme served as a receptor for divalent cations in bacterial chemotaxis (95). The enzyme was implicated in the phenomenon of exocytosis in Paramecium (96) and it may play a role in secretory processes in mast cells (97). Ecto-ATPases may function as regulatory components in excitable tissues where nucleotides act as transmitters (98) or as modulators of excitability thresholds (99). Adenylates and related compounds are powerful agonists when applied to the cell surface and they often induce transient change in membrane

permeability (100). The ecto-ATPase of Ehrlich ascites tumor cells has been linked with the regulation of cell motility and volume (101). It has been suggested that a contractile protein is present in plasma membrane of Ehrlich cells which respond to external ATP. In this regard, myosin and actin like contractile proteins have been reported to reside in the plasma membranes of various cell types, and their function in plasma membrane contractility has been suggested. However, the relationship between plasma membrane contractile proteins and ecto-ATPase still remains unclear.

A number of investigators implicated the role of ecto-ATPase in the entry of divalent cations into cells. Parish and Weibel (102) recently demonstrated that an ecto-ATPase in Dictyostelium Discodium cells, when inhibited by suramin, resulted in depressed Ca^{2+} influx into these cells. These authors also noted that hexokinase and apyrase, which split exogenous ATP, resulted in decreased Ca^{2+} influx. Landry and Lehninger (103) also noted that extracellular ATP was necessary to promote Ca^{2+} influx into Ehrlich ascites cells. These authors also indicated the involvement of an ecto-ATPase in the Ca^{2+} influx phenomenon. A divalent cation dependent ecto-ATPase activity was recently observed in cultured hamster cardiac cells and also rat heart myocytes (104). Cytochemical localization of the divalent cation dependent ATPase at the sarcolemma of cardiac cells was also demonstrated (105).

Previous work on the rat heart sarcolemmal divalent cation ATPase, has revealed that the enzyme is activated by mM concentration of divalent

cations; the order of potency being $Ca^{2+} > Mg^{2+} > Mn^{2+} > Co^{2+} > Ba^{2+} > Sr^{2+}$. The K_a value of the membrane bound enzyme for Ca^{2+} and Mg^{2+} was found to be about 0.6 mM and 0.8 mM respectively. The enzyme was found to have a k_m value of about 0.3 mM for ATP. Treatment of sarcolemma with phospholipases showed a marked decrease in ATPase activity measured in the presence of Mg^{2+} whereas the ATPase activity in the presence of Ca^{2+} was only slightly effected. Proteolytic digestion of the sarcolemmal membranes with trypsin, resulted in stimulation of the ATPase activity in the presence of Ca^{2+}, whereas the Mg^{2+} activated enzyme activity was unaffected. Effects of detergents such as deoxycholate on the sarcolemmal ATPase, showed that activity in the presence of Mg^{2+} was inactivated, at rather mild treatments, whereas the activity in the presence of Ca^{2+}, was still detectable even at the most harsh treatments (106). The results suggest that the two cations, Ca^{2+} and Mg^{2+}, act at different sites on the ATPase molecule, or that there are two types of ATPases present, i.e. Ca^{2+} ATPase and a Mg^{2+} ATPase.

Although the exact function of the sarcolemmal divalent cation dependent ATPase activity is unknown at present, this activity was reported to be depressed in different types of failing hearts (1). Pharmacological agents such as quinidine, lidocaine and procaine (107) as well as propranolol (108) which depressed contractile force, decreased the activity of the sarcolemmal ATPase. Furthermore, divalent cations including Ni^{2+}, Co^{2+} and Mn^{2+}, which inhibited the entry of

Ca^{2+} into the myocardial cell, decreased heart sarcolemmal Ca^{2+} ATPase activity (109). Phosphorylation of sarcolemma by cyclic AMP - protein kinase, which is known to increase Ca^{2+} entry, increased sarcolemmal ATPase activity (110). It was thus suggested that the divalent cation ATPase activity, may be involved with opening "Ca^{2+} channels" in the sarcolemma to allow Ca^{2+} entry into the cardiac cell (111). At this stage the biochemical nature and the physiological role of the divalent cation ATPase in sarcolemma is unclear. Since no attempt has been made to solubilize and purify the heart sarcolemmal divalent cation ATPase, it is the purpose of this study to isolate this enzyme system in a purified form. It is hoped that the achievement of this objective will open up a way for understanding the physiological significance of this important enzyme system in cellular function.

III. MATERIALS AND METHODS

Healthy albino rats or mongrel dogs were used in this study. All chemicals used were of analytical grade. Trypsin inhibitor (from soybean), trypsin (pancreas), cyclic 3', 5' adenosine monophosphate, adenosine triphosphate and protein kinase (from beef heart) were obtained from Sigma. Sephadex G-200 and Phenyl-Sepharose CL-4B was obtained from Pharmacia Fine Chemicals. Purified calmodulin and calmodulin affinity column was a kind gift from Dr. Jerry Wang of the Biochemistry Department of this University.

A. Isolation of Heart Sarcolemma.

1. Rat heart sarcolemmal preparation.

Rats were decapitated and their hearts quickly removed and placed in ice-cold 10 mM Tris-HCl buffer, pH 7.4. The ventricles were washed thoroughly, diced with a pair of scissors, homogenized with 10 vol of 10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA in a Waring blender for 1 min (30 s x 2 with an interval of 30 s). The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 1000 g for 10 min. The sediment was suspended in 20 to 25 vol of 10 mM Tris-HCl buffer, pH 7.4 and stirred in the cold room for 30 min and centrifuged at 1000 g for 10 min. The residue was then suspended in 20 to 25 vol of the Tris-HCl buffer, pH 8.0 stirred for 30 min and centrifuged at 1000 g for 10 min. The sediment was suspended in 10 mM Tris-HCl buffer, pH 7.4, stirred for 30 min and centrifuged at 1000 g for 10 min; this step was then repeated again. The sediment was suspended in 20 to 25 vol of 10 mM

Tris-HCl, pH 7.4, extracted with 0.4 M LiBr for 45 min and centrifuged at 1000 g for 10 min. This sediment was then suspended in 10 mM Tris-HCl, pH 7.4, stirred for 10 min and centrifuged at 1000 g for 10 min. The sediment was further extracted with 0.6 M KCl containing 10 mM Tris-HCl, pH 7.0 washed thoroughly and employed for biochemical studies. All the above steps were performed at 0 to 4°C. This procedure is essentially similar to that described earlier (112). The activities of the enzymes such as adenylate cyclase, $Na^+ - K^+$ ATPase, ouabain sensitive $Na^+ - K^+$ ATPase, Ca^{2+} - stimulated Mg^{2+} - dependent ATPase were examined as described earlier (112 - 114). The calcium binding activity of the sarcolemmal fraction in the presence of ATP was investigated as described elsewhere (113). The membrane fraction was also examined electron microscopically as described by Anand et al. (85).

2. Dog heart sarcolemmal preparation.

The isolation of sarcolemma from dog left ventricle was essentially, according to the procedure described for rat hearts, except that the extraction with 0.6 M KCl was not employed.

B. Treatment of Heart Sarcolemma.

1. Trypsin treatment.

About 260 - 300 mg of sarcolemmal protein was suspended at a concentration of 2 mg/ml in a medium of composition, 20 mM KCl and 50 mM Tris HCl buffer, pH 7.4, at room temperature. Trypsin at a concentration of 0.1 mg/mg of sarcolemmal protein was added, the

mixture stirred for 15 min., and the reaction terminated by the addition of trypsin inhibitor (0.3 mg/mg of protein). The trypsin treated fraction was centrifuged at 100,000 g for 45 min at 4°C. The supernatant was collected and stored at -20°C.

2. Triton X-100 treatment

The pellet from 100,000 g spin was suspended in 30 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 1% Triton X-100, by homogenization with a "glass-glass" homogenizer. The homogenized material was then transferred into 3 glass test tubes, about 10 ml in each test tube. Each test tube was then sonicated, with a probe sonicator at a setting of '40' on the high scale. About 3 - 4, 3 min bursts per 10 ml of sample were used. During sonication the temperature of the sample was allowed to rise slightly above room temperature, and then immediately cooled to 0 - 4°C. After sonication the samples from all 3 test tubes were pooled and centrifuged at 100,000 x g for 45 min at 4°C. The yellow-brown supernatant was collected, and the pellet was suspended in 10 ml of 1 mM Tris-HCl, pH 7.4 buffer.

3. Ammonium sulphate treatment

The yellow-brown supernatant was transferred to a beaker and stirred with $(NH_4)_2\ SO_4$ (0.5 g/ml) until the salt dissolved. The mixture was then transferred into centrifuge tubes and left in ice for 1 hr. in the cold room. The centrifuge tubes were then spun at 18,000 rpm,

pH 7.4 buffer, which appeared as a yellowish-brown solution. This solution was transferred into dialysis tubing, and dialysed against 4 L of 50 mM Tris-HCl, pH 7.4 buffer for 24 hr. This sample was then subjected to column chromatography.

C. Column Chromatography

1. Phenyl-Sepharose (hydrophobic) column

The yellowish-brown, dialysed sample in 50 mM Tris-HCl, pH 7.4 and 1 M $(NH_4)_2SO_4$ was subjected to hydrophobic interaction chromatography on phenyl groups attached to agarose gel Sepharose CL-4B.

A column (1 x 8 cm) was packed with phenyl Sepharose; samples of protein exhibiting ATPase activity were applied to phenyl Sepharose column in a buffer containing 50 mM Tris-HCl, pH 7.4 and 1 M $(NH_4)_2SO_4$. The protein was eluted from this column with double distilled water, and then with 1% Triton X-100. Samples of protein exhibiting ATPase activity obtained from the phenyl-Sepharose column were applied to the Sepharose-6B column.

2. Sepharose-6B column

Gel-filtration on a Sepharose-6B column (2 cm x 90 cm) which had been equilibrated with 50 mM Tris-HCl, pH 7.4 buffer containing 1% Triton X-100 was performed. The protein was eluted with the equilibrating buffer at the rate of 10 to 12 ml/hr. The fractions exhibiting ATPase activity obtained from this column were pooled and lyophillized.

3. Sephadex G-200 column

The lyophillized material was redissolved in 3 mls of 1 mM Tris-HCl, pH 7.4 buffer and applied to a Sephadex G-200 column (2 x 50 cm) equilibrated with 50 mM Tris-HCl, pH 7.4 buffer. The Protein from the column was eluted with the equilibrating buffer and the fractions exhibiting ATPase activity were analysed for purity and characterization.

4. DEAE cellulose column

Fractions exhibiting ATPase activity obtained from the Sepharose-6B column were pooled and applied to a DEAE cellulose column (2.5 x 25 cm) which had been equilibrated with 50 mM Tris-HCl buffer, pH 7.4. The protein was eluted with a linear gradient of 50 - 500 mM Tris-HCl (pH 7.4). The column was then washed with 3 M Tris-HCl, pH 7.4.

5. Calmodulin affinity column

Purified protein sample exhibiting ATPase activity was applied to a calmodulin affinity column (1 x 6 cm) in a Tris-HCl buffer, pH 7.0 containing 2 mM $CaCl_2$. The protein was eluted from the column by washing with two column bed volumes of the sample buffer, then another two column bed volumes consisting of the sample buffer containing 0.2 M NaCl. The column is then washed with the sample buffer containing 0.1 mM EGTA but without $CaCl_2$.

D. Purification of Myosin from Dog Heart Ventricles

Myosin was isolated from dog heart ventricles according to the

method described by (115). Antiserum to purified myosin and $Na^+ + K^+$ ATPase was prepared by the Antibodies Inc., Davis, California.

E. Determination of Enzyme activities

1. Mg^{2+} dependent ATPase

The Mg^{2+} dependent ATPase activity was assayed in a total volume of 1 ml containing 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, 4 mM $MgCl_2$ 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 were centrifuged and the Pi in the clear supernatant was determined. The ATP hydrolysis that occurred in the absence of Mg^{2+} was subtracted in order to calculate the activity due to Mg^{2+} dependent ATPase. The estimation of Pi was carried out by the method of Taussky and Shorr (115).

2. Ca^{2+} dependent ATPase

The Ca^{2+} ATPase activity was assayed in a total volume of 1 ml containing 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, 4 mM $CaCl_2$ and 4 mM Tris-ATP. After 5 min of pre-incubation at 37°C with the enzyme 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 were centrifuged and Pi in the clear supernatant was determined. The ATP hydrolysis that occurred in the absence of Ca^{2+} was subtracted in order to calculate the activity due to Ca^{2+} stimulated ATPase.

F. Gel Electrophoresis

1. Electrophoresis of active enzyme

Electrophoresis of the native enzyme was carried out using 7.5% acrylamide gels, Tris-HCl (pH 8.0) as gel buffer and Tris-glycine (pH 8.9) as electrode buffer according to the method described by Davis (117). The gels were run in duplicate one set being stained with 0.25% Coomassie brilliant blue and destained in 7% acetic acid. The other gel was sliced into 2 mm strips, suspended in 50 mM Tris-HCl buffer (pH 7.4) and tested for ATPase activity.

2. Electrophoresis in sodium dodecyl sulfate

This was performed with 10% acrylamide gels according to Weber and Osborn (118). Samples were prepared by heating at 100°C for 3 min in the presence of 19% sodium dodecyl sulfate, 5% mercaptoethanol, 0.5 M sucrose and 0.01% bromphenol blue in a final volume of 0.1 ml. Electrophoresis was run at 8 mA per tube. The gels were fixed in a solution containing 7.5% trichloroacetic acid and 50% methanol, stained with 0.25% coomassie brilliant blue, and destained with 7.5% acetic acid and 5% methanol. In some cases the system of Laemmli was employed for SDS gel electrophoresis (119). Standards used were thyroglobulin (Mr = 330,000), phosphorylase b (94,000), bovine serum albumin (Mr = 67,000), catalase (Mr - 60,000), lactate dehydrogenase (Mr = 36,000), and Ferritin (Mr = 18,500).

G. Determination of Molecular Weight by Gel Filtration

The molecular weight of the native protein was estimated relative to proteins of known molecular weight by gel filtration chromatography

on a Sephadex G-200 superfine column (2.5x 50 cm) which was equilibrated and eluted with 50 mM Tris-HCl buffer, pH 7.5, at 4°C. The marker proteins and their molecular weights were: chymotrypsinogen (Mr = 25,000), ovalbumin (Mr = 43,000) and albumin (Mr = 67,000). aldolase (Mr = 158,000), catalase (Mr = 232,000) and ferritin (Mr = 440,000).

H. Determination of Protein Concentration

Protein was estimated by the method of Lowry et al. (120) using bovine serum albumin as a standard. In some cases absorbance at 280/260 and the Bio-Rad protein determination method was used (121).

I. Phosphorylation of ATPase

For phosphorylation studies 100 µl sample of purified preparation was incubated with equal volume ATP (specific activity, 1000 dpm/p mole) and 125 units of the cyclic-AMP dependent catalytic subunit of protein kinase. The final concentration of ATP in the assay media was 0.25 mM and 4.5 mM Mg^{2+}. The reaction was stopped by spotting 25 µl aliquots of the assay mixture onto a filter paper and the protein precipitated by filter-disc method of Butcher (122).

IV. RESULTS

A. Characterization of Heart Sarcolemma

As reported earlier (113, 114) the sarcolemmal fraction prepared by the hypotonic shock-LiBr treatment contained negligible amounts (2 to 4%) of contamination by other subcellular organelles such as mitochondria, sarcoplasmic reticulum and myofibrils. Furthermore, unlike mitochondria and fragments of the sarcoplasmic reticulum (microsomes), this preparation did not show any ATP-dependent calcium binding and unlike microsomes and myofibrils, it did not exhibit any Ca^{2+} - stimulated Mg^{2+} - dependent ATPase activity. On the other hand, this membrane fraction from rat heart showed high specific activities of adenylate cyclase (350 to 450 pmol cyclic AMP/min/mg protein) and Na^{+} - K^{+} ATPase (12 to 16 µmol Pi/h mg protein); these enzyme activities in the membrane preparation were 7 to 9 fold higher than those present in the heart homogenate. The Na^{+} - K^{+} ATPase activity of this membrane preparation was inhibited by 80 to 90% by 2 mM ouabain. In addition it contained a divalent cation dependent ATPase activity, which catalysed ATP hydrolysis in the presence of Ca^{2+} or Mg^{2+}. These enzyme activities were not altered by further extraction of the membranes with 0.6 M KCl, which is considered to remove myofibrillar proteins. By using lanthanum labelling technique, it has been reported earlier that this preparation is of the cell membrane origin (114). The electron microscopic examination of the membrane preparation revealed the presence of membrane sacs of various shapes and sizes with a

fuzzy coat of basement membrane on the outer side of these vesicles (114).

B. Dog Heart Sarcolemmal Divalent Cation Dependent ATPase

1. Solubilization

The dog heart sarcolemmal membranes exhibited a divalent cation dependent ATPase activity which was about 12 μmoles Pi/mg/hr in the presence of 4 mM Mg^{2+} and about 8 μmoles Pi/mg/hr in the presence of 4 mM Ca^{2+}. Upon trypsin treatment of sarcolemma, the divalent cation ATPase activity was retained in the 100,000 x g pellet, whereas a Ca^{2+} dependent ATPase activity released into the supernatant (84). The divalent cation ATPase was solubilized from the 100,000 x g pellet by sonication in 1% TX-100. Prior treatment of the membranes with trypsin was found essential for the solubilization of the enzyme. As shown in Table I, the solubilization of the ATPase was dependent on the temperature of sonication. When the sonication was carried out at 0° - 4°C, about 30% of protein was solubilized. The solubilized fraction contained the ATPase activity which retained ion dependency for both Mg^{2+} (31 μmoles Pi/mg/hr) and Ca^{2+} (8 μmoles Pi/mg/hr). However, when the sonication was carried out at 37°C, about 66% of the protein was solubilized, but the Ca^{2+} ion dependent ATPase activity of the solubilized enzyme was reduced to about 1 μmol Pi/mg/hr. Sonication at a temperature of 20 - 25°C was found to be suitable for adequate solubilization and retention of divalent cation dependency of the ATPase (Table II).

TABLE I. Effect of Temperature on Solubilization of Divalent Cation Dependent ATPase from Dog Heart Sarcolemma.

	Total Activity µmoles Pi/hr		Protein mg	Specific Activity	
	4 mM Ca^{2+}	4 mM Mg^{2+}		4 mM Ca^{2+}	4 mM Mg^{2+}
1. Sonication at 0 - 4° C	213	903	30	7.1	30.1
100,000 x g pellet	92	693	19.1	4.8	36.3
Supernatant	64	259	8.3	8.1	31.2
2. Sonication at 37° C	177	1011	30	5.9	33.7
100,000 x g pellet	17.4	341	7.9	2.2	43.1
Supernatant	22.3	664	20.3	1.1	32.7

TABLE II. Purification of Divalent Cation Dependent ATPase from Dog Heart Sarcolemma.

	Total Activity µmoles Pi/mg		Protein mg	Specific Activity µmoles Pi/mg/h		Yield of enzyme %	
	4 mM Mg^{2+}	4 mM Ca^{2+}		4 mM Mg^{2+}	4 mM Ca^{2+}	4 mM Mg^{2+}	4 mM Ca^{2+}
Sarcolemma	1016.8	615	82	12.4	7.5	100	100
100,000 x g pellet after trypsin treatment	1334	188.1	28.5	46.8	6.6	132	30.6
100,000 x g supernatant obtained after sonication at 25 °C	856.5	75.9	18.5	46.3	4.1	84.2	12.3
Ammonium Sulphate precipitate	554.7	65.2	12.3	45.1	5.3	54	10.6
Sepharose-6B column	360	75	6	60.0	12.5	35	12.2
DEAE-cellulose column	11.1	2.4	0.3	37.0	8.0	1.1	0.40

2. Isolation

The solubilized material obtained after sonication was treated with ammonium sulphate; about 50% Mg^{2+} - dependent and 10% Ca^{2+} dependent enzyme activity was recovered in 70% ammonium sulphate precipitate (Table II). Gel filtration of the enzyme preparation on Sepharose-6B column resulted in the elution of ATPase with a major protein peak immediately after the void volume (Fig. 1). About 35% Mg^{2+} - dependent and 12% Ca^{2+} - dependent ATPase activity was recovered on this column. Fractions exhibiting ATPase activity were pooled and applied to a DEAE cellulose column and the elution profile is shown in Fig. 2. The enzyme was eluted from the column with 3 M Tris-HCl buffer, pH 7.4, and was separated from the major protein peak. About 0.5 to 1% of the total ATPase activity was recovered on this column. The enzyme was found to be extremely labile under the conditions as total loss of the activity was observed within 24 hrs of isolation. Both total and specific activity of the ATPase were markedly reduced after the DEAE cellulose column step.

3. Properties of the enzyme

Purity- Due to instability of the ATPase enzyme, investigation on the purity of the preparation could not be adequately conducted. Furthermore, the enzyme preparation did not appear to enter the polyacrylamide gels, under non-denaturing or S.D.S. gel electrophoresis. This was probably due to the association of phospholipids with the proteins, which possibly interfere with the migration of the proteins.

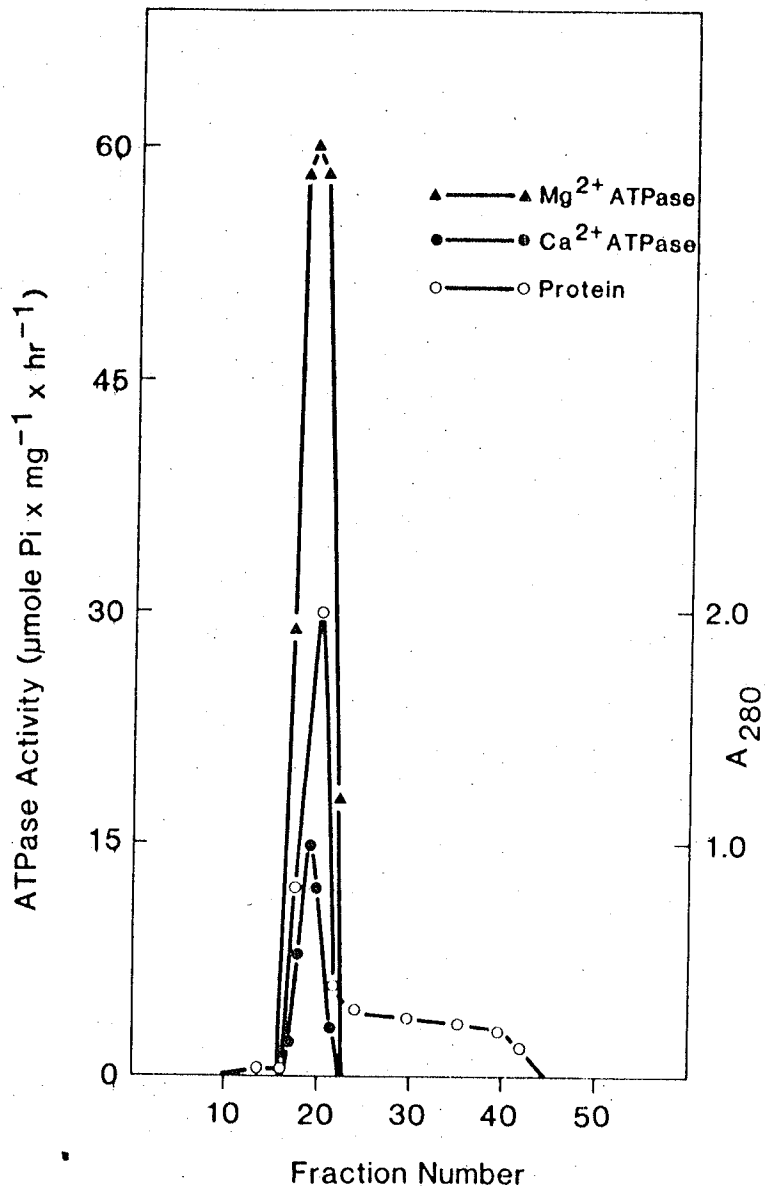

FIGURE 1. Column chromatography on Sepharose-6B of solubilized ATPase fraction from dog heart sarcolemma. The TX-100 solubilized sarcolemmal protein sample was applied and eluted with 50 mM Tris-HCl buffer, pH 7.4, containing 1% TX-100. 0.1 ml aliquots of the eluate were used to determine ATPase activity.

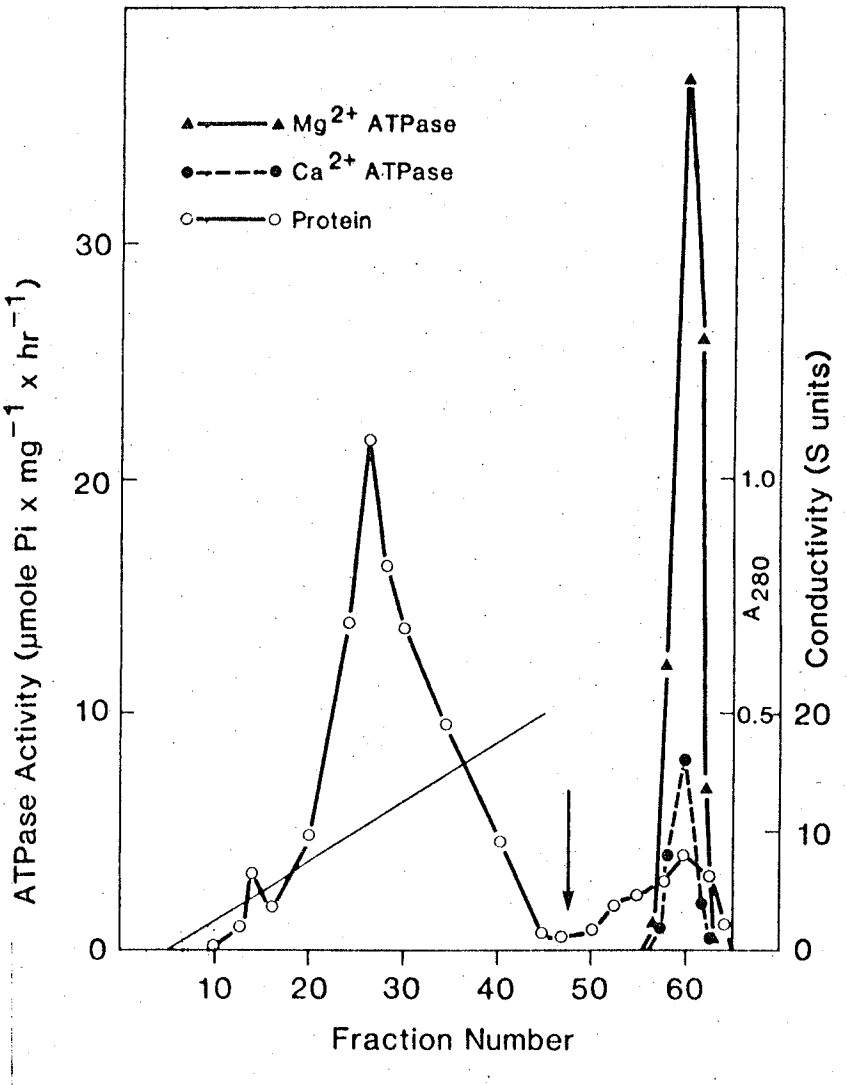

FIGURE 2. Elution profile on DEAE cellulose column of pooled fractions exhibiting ATPase activity obtained from Sepharose-6B column. The protein was first eluted with Tris-HCl linear gradient (50 - 500 mM) and then washed with 3 M Tris-HCl buffer, pH 7.4, indicated by the arrow.

Reactivity with myosin antibodies and $Na^+ + K^+$ ATPase antibodies

To investigate whether the divalent cation dependent ATPase was different from myosin and $Na^+ + K^+$ ATPase, the reactivity of the solubilized ATPase with dog heart myosin antiserum and $Na^+ + K^+$ ATPase antiserum was investigated (Table III). The results indicate that the divalent cation dependent ATPase does not show any cross-reactivity with either myosin antiserum or $Na^+ + K^+$ ATPase antiserum.

Effect of inhibitors

As shown in Table IV, the enzyme was insensitive to ouabain, verapamil, oligomycin and vanadate.

The solubilized ATPase did not exhibit any Ca^{2+} stimulated Mg^{2+} dependent ATPase activity. The monovalent cations such as Na^+ and K^+ were without effect on the ATPase activity.

C. Rat Heart Sarcolemmal Divalent Cation Dependent ATPase

1. Solubilization

The rat heart sarcolemmal membranes exhibited a divalent cation dependent ATPase activity of about 32 μmoles Pi/mg/hr in the presence of Ca^{2+} and about 27 μmoles Pi/mg/hr in the presence of Mg^{2+}. Trypsin treatment of sarcolemma resulted in the release of a Ca^{2+} - dependent ATPase in the supernatant (84), and the retention of a divalent cation dependent ATPase in the 100,000 x g pellet. As for dog sarcolemmal divalent cation dependent ATPase, the rat heart sarcolemmal enzyme was solubilized by sonication in TX-100; the solubilization was dependent on the temperature of sonication (Table V). Sonication at 0 - 4°C resulted in about 35% solubilization of protein. The

TABLE III. Reactivity of Myosin Antibodies and $Na^+ + K^+$ ATPase Antibodies with Solubilized Dog Sarcolemmal Divalent Cation Dependent ATPase.

	Divalent cation ATPase µmole Pi/mg/hr		Myosin ATPase µmoles Pi/mg/hr	$Na^+ + K^+$ ATPase µmoles/Pi/mg/hr
	4 mM Ca^{2+}	4 mM Mg^{2+}	10 mM Ca^{2+}	
Control	3.8	61.3	17.5	7.1
Myosin-antisera	3.9	58.9	4.2	6.3
$Na^+ + K^+$ ATPase antisera	3.4	57.2	15.9	1.5

About 20 µg of enzyme protein was preincubated for 5 min. with 100 µl antiserum (1 : 5 dilution), and the ATPase activity determined as indicated in "Methods".

TABLE IV. Effect of Various Inhibitors on Divalent Cation ATPase

Inhibitor	Divalent ATPase Activity µmoles Pi/mg/hr	
	4 mM Mg^{2+}	4 mM Ca^{2+}
Control	43.6	5.1
Ouabain (2 mM)	41.2	4.9
Verapamil (5 µM)	44.7	5.0
Oligomycin (50 µM)	42.8	4.9
Vanadate (100 µM)	40.7	4.8

Solubilized enzyme (20 µg) was incubated in the presence of 4 mM Ca^{2+} or Mg^{2+} and the given concentration of inhibitor. ATPase assay was carried out as described in the text. The results are average of two different preparations.

TABLE V. Effect of Temperature on Solubilization of Divalent Cation Dependent ATPase from Rat Heart Sarcolemma.

	Total Activity µmoles Pi/hr		Protein mg	Specific Activity µmoles Pi/mg/hr	
	4 mM Ca^{2+}	4 mM Mg^{2+}		4 mM Ca^{2+}	4 mM Mg^{2+}
1. Sonication of trypsin treated preparation at 0 - 4° C	1715	1580	45	38.1	35.1
100,000 x g pellet	1168	1098	28	41.7	39.2
Supernatant	670	512	17	39.4	30.1
2. Sonication of trypsin treated preparation at 37° C	1904	464	45	42.3	10.3
100,000 x g pellet	72.8	15.6	13	5.6	1.2
Supernatant	1251	262	32	39.1	8.2

solubilized fraction contained ATPase which retained ion dependency for both Ca^{2+} (39 μmoles Pi/mg/hr) and Mg^{2+} (30 μmoles Pi/mg/hr). When sonication was carried out at 37°C, about 70% of protein was solubilized but the Mg^{2+} dependent activity of the solubilized ATPase was reduced to about 8 μmol Pi/mg/hr. As for the dog heart sarcolemmal ATPase, a temperature of 20 - 25°C was found to be most suitable for solubilization of the divalent cation ATPase activity.

2. Purification

The purification of the divalent cation dependent ATPase from rat heart sarcolemma is shown in Table VI. About 12% of total divalent cation dependent ATPase activity was recovered in 70% ammonium sulphate precipitate. Hydrophobic chromatography on a phenyl Sepharose column of the ATPase resulted in the elution of the enzyme activity with two different reagents. The first fraction was obtained by elution with water, whereas the second fraction of the enzyme was obtained by eluting with 1% TX-100 (Fig. 3). About 6% total ATPase activity was recovered from this column. The fractions exhibiting ATPase activity were pooled and applied to a gel-filtration, Sepharose-6B column (Fig. 4). About 3% total ATPase activity was recovered on this column and the fractions exhibiting the enzyme activity were pooled and applied to a Sephadex-G200 column (Fig. 5). About 0.6% total activity of the ATPase was recovered, with a specific activity of 340 μmoles Pi/mg/hr in the presence of Ca^{2+} and 220 μmoles Pi/mg/hr in the presence of Mg^{2+}. An overall purification factor of about 8 - 10 fold was achieved by this procedure.

TABLE VI. Purification of Divalent Cation Dependent ATPase from Rat Heart Sarcolemma.

	Total Activity µmoles Pi/hr		Protein mg	Specific Activity µmoles Pi/mg/hr		Yield enzyme %	
	4 mM Ca^{2+}	4 mM Mg^{2+}		4 mM Ca^{2+}	4 mM Mg^{2+}	4 mM Ca^{2+}	4 mM Mg^{2+}
Sarcolemma	18,900	16,680	600	31.5	27.8	100	100
100,000 x g pellet obtained after trypsin treatment	12,404	11,312	280	44.3	40.4	65.6	67
100,000 x g supernatant obtained after sonication in TX-100	4,966	3,913	130	38.2	30.1	26.3	23.4
Ammonium sulphate precipitate	2,499	1,876	70	35.7	26.8	13.2	11.3
Phenyl sepharose column	1202.4	650.4	24	50.1	27.1	6.4	3.8
Sepharose-6B column	556.2	441.3	3	185.4	147.1	2.9	2.6
Sephadex-G-200 column	136	88	0.4	340	220	0.72	0.53

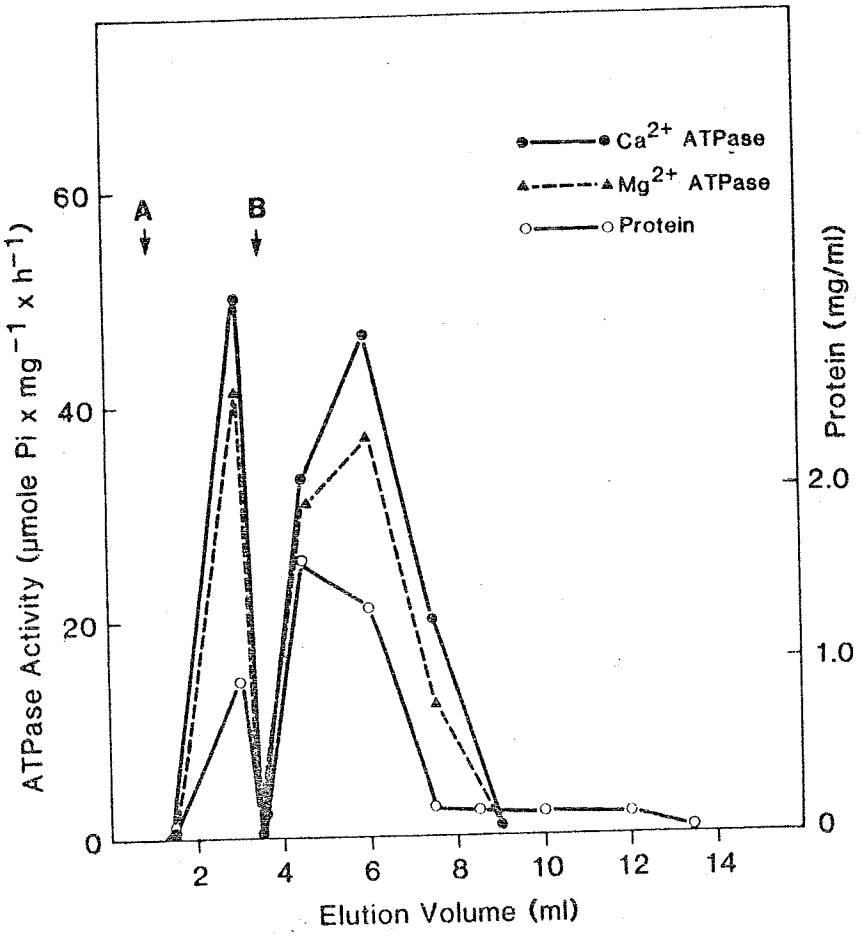

FIGURE 3. Hydrophobic chromatography of the solubilized ATPase on a phenyl Sepharose column. 2 mls of sample were applied to the column as described in Methods and then eluted with A(H_2O) and B(1% TX-100). The flow rate was about 8 mls/hr.

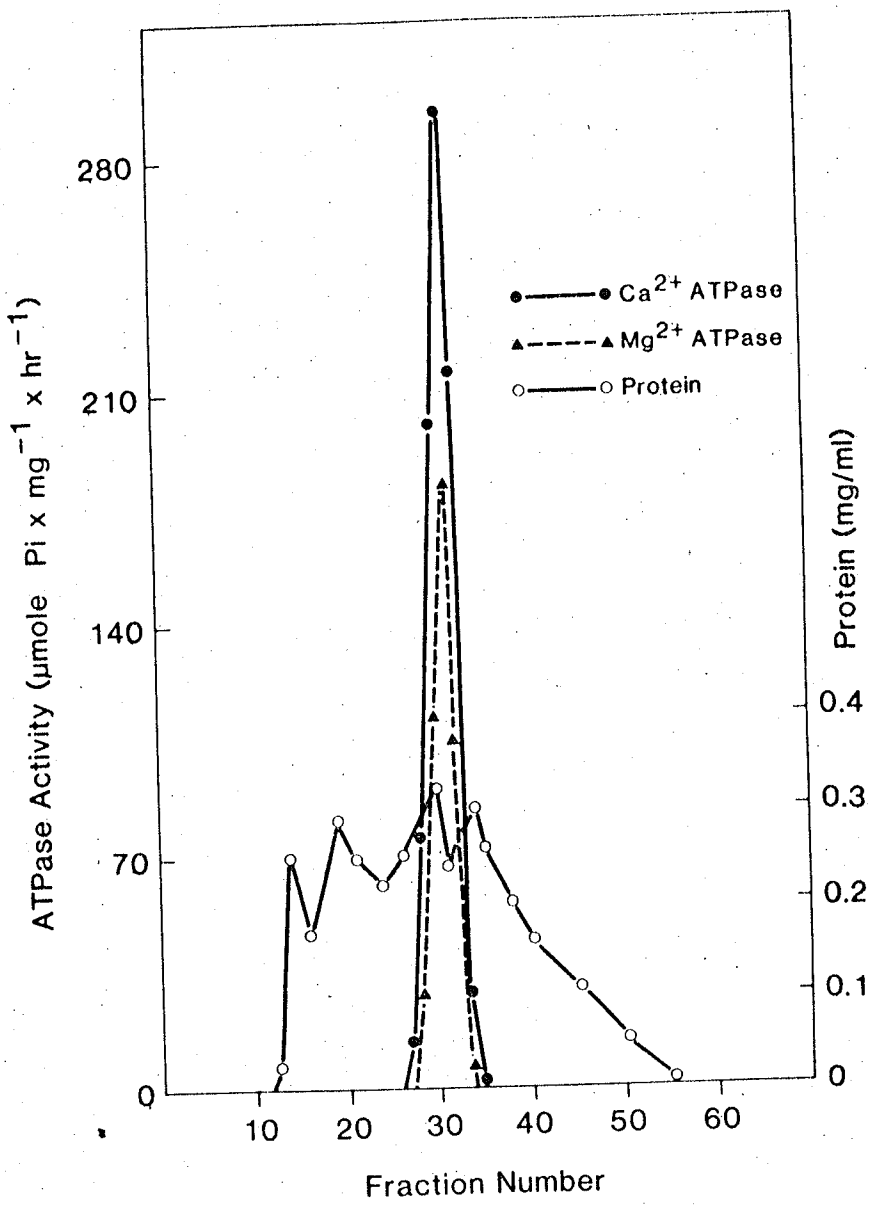

FIGURE 4. Column chromatography on Sepharose-6B of pooled fractions exhibiting ATPase activity obtained from the hydrophobic column. About 60 drops/tube were collected at a flow rate of 10 mls/hr.

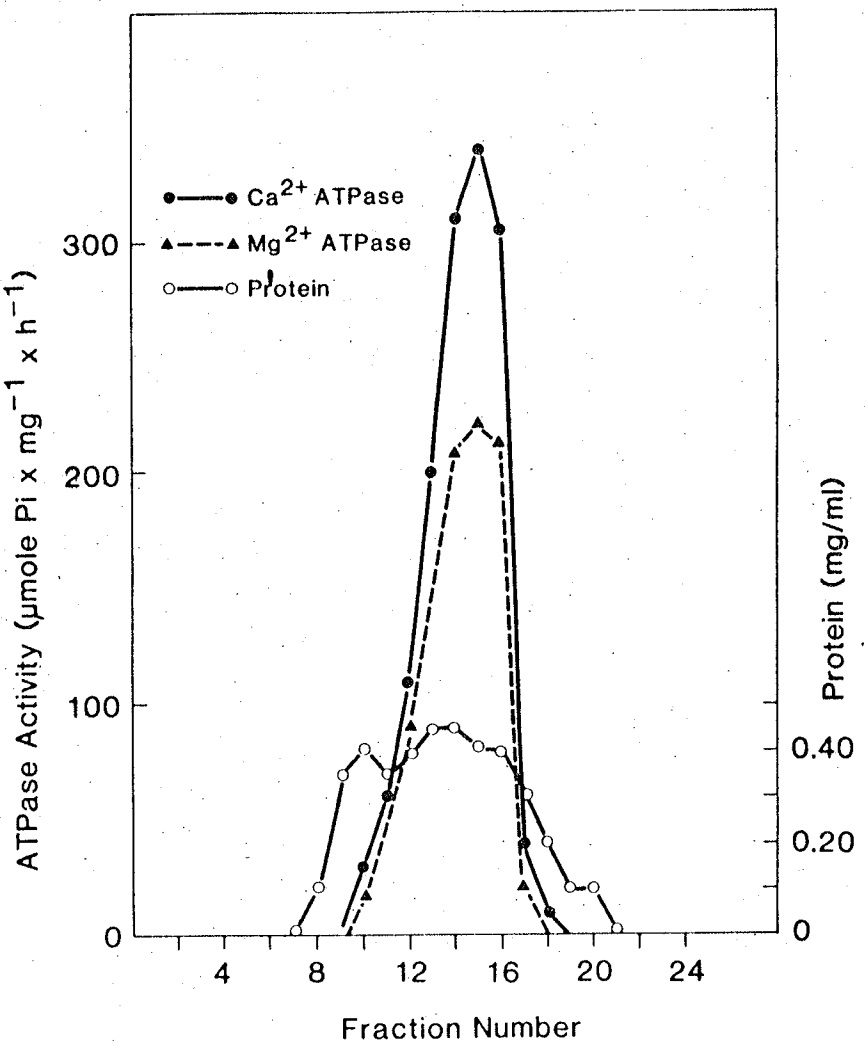

FIGURE 5. Gel filtration on Sephadex-G200 column of pooled fractions exhibiting ATPase activity obtained from Sepharose-6B column.

3. Properties of the enzyme

Purity - Fig. 6 shows that an enzyme preparation obtained from the Sepharose - 6B column with a specific activity of about 200 µmol Pi/mg/hr contained two protein bands in nondenaturing gel electrophoresis. The divalent cation dependent ATPase activity was associated with the protein band which had an Rf value of 0.12 (Fig. 6). The enzyme preparation obtained from the Sephadex - G200 column, with a specific activity of about 300 µmoles Pi/mg/hr contained a single major protein band in nondenaturing gel electrophoresis (Fig. 7); the ATPase activity was associated with the protein band.

Subunit Structure - For determination of the subunit structure, the enzyme was first electrophoresed in the absence of sodium dodecyl sulfate and the band corresponding to the ATPase activity was cut out and used for gel electrophoresis in the presence of sodium dodecyl sulfate. The purified enzyme revealed 3 major subunits with molecular weights estimated as 90,000, 80,000 and 67,000 (Fig. 8). The migration of the enzyme subunits under different polyacrylamide concentrations in the presence of S.D.S. was also investigated (Fig. 9).

Estimation of Molecular Weight - The elution volume of the purified enzyme was similar to that of catalase on Sephadex G-200 column, about 240,000 (Fig. 10). This size is consistent with the subunit structure observed in S.D.S. polyacrylamide gels.

Stability - The purified enzyme, was stable for about 7 days at -15°C. Addition of the reducing agent dithiothreitol did not improve the stability of the ATPase.

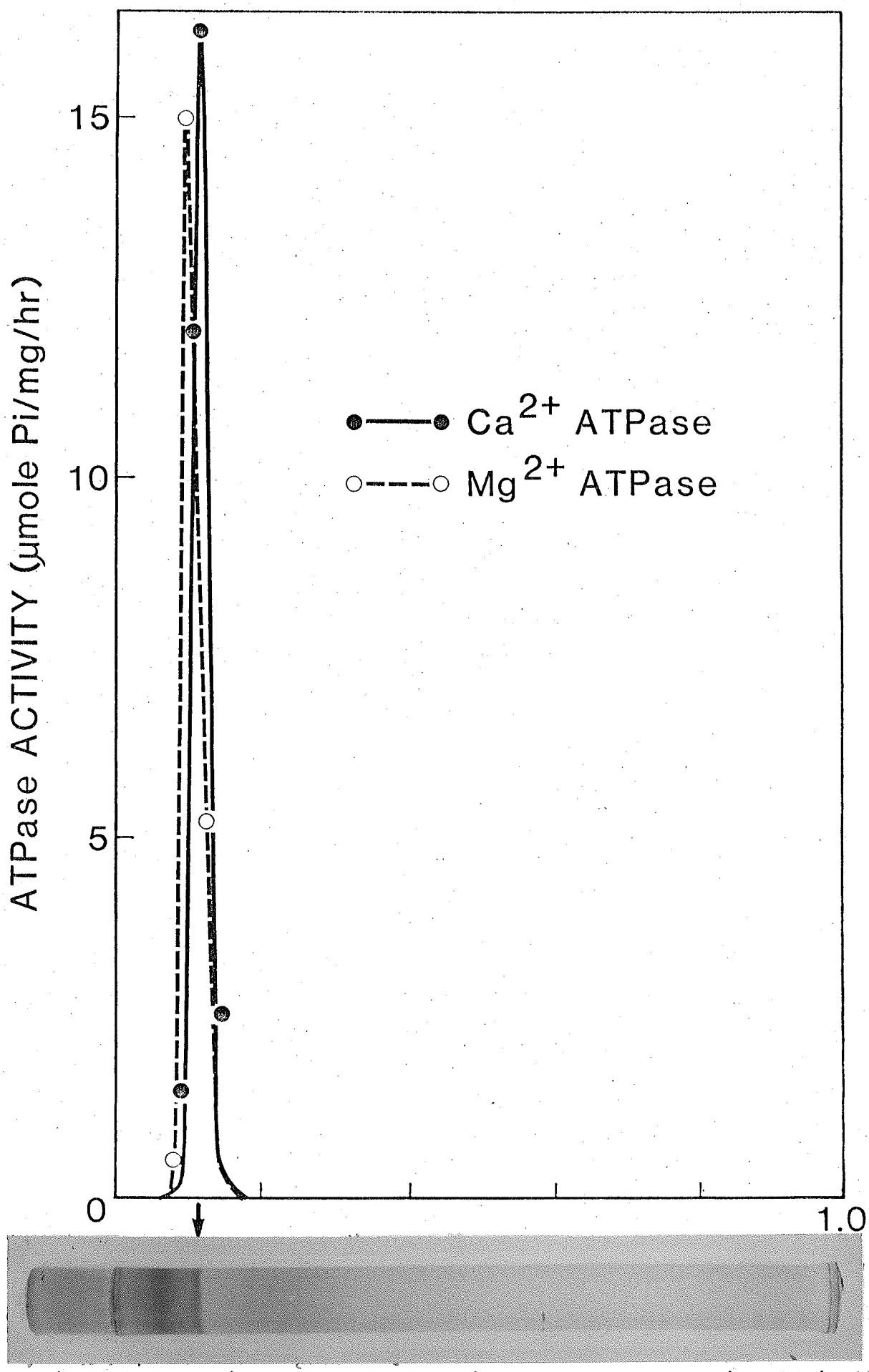

FIGURE 6. Polyacrylamide gel electrophoresis of the ATPase fraction obtained from the Sepharose-6B column (60 μg) was applied to 7.5% polyacrylamide gels.

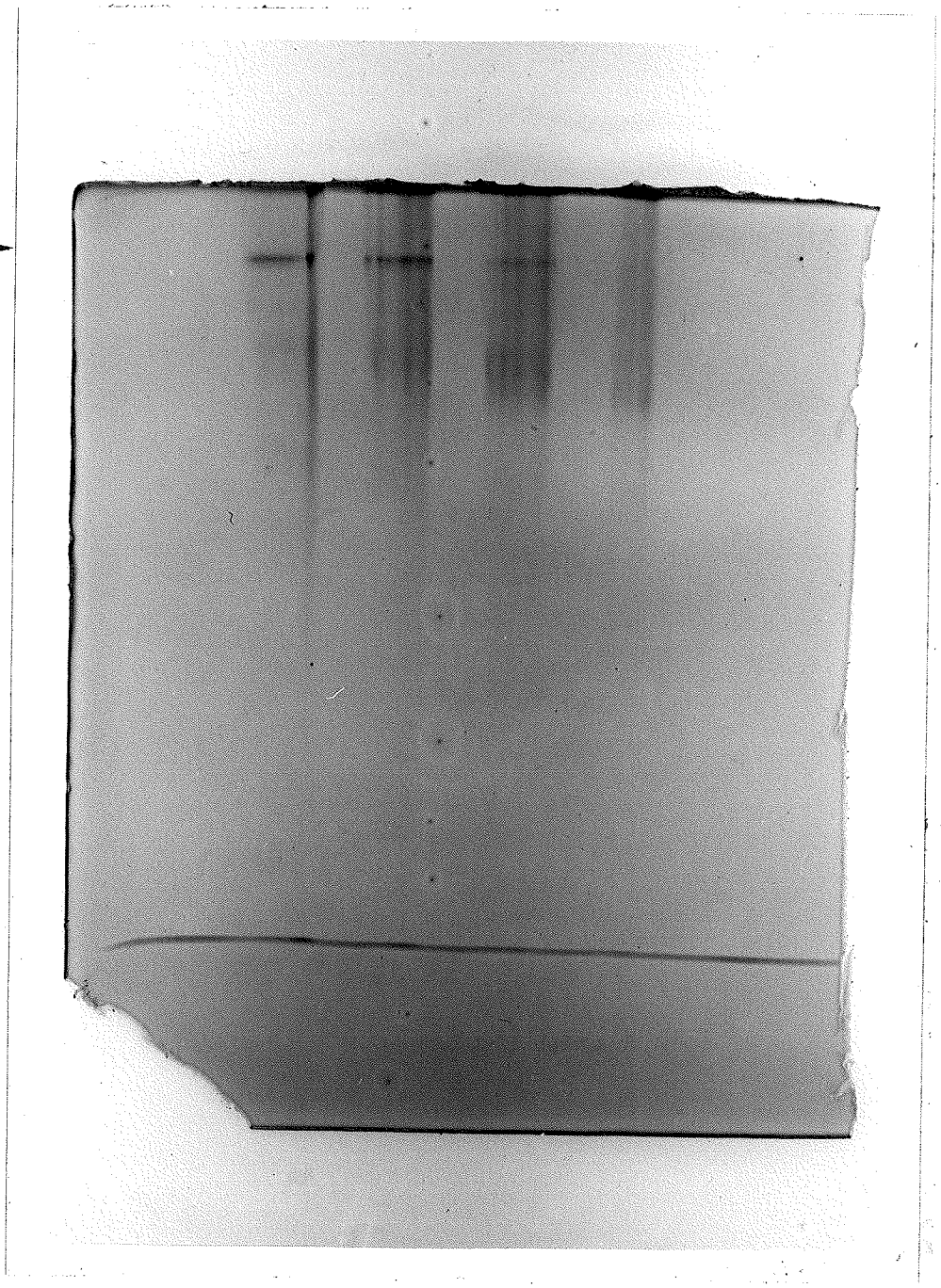

FIGURE 7. Polyacrylamide gel electrophoresis of the purified ATPase. 7 µg of the native enzyme was applied to 7.5% polyacrylamide slab gels of 1.5 mm thickness. The ATPase activity was found to be associated with the major protein band and was assayed as described under "Methods".

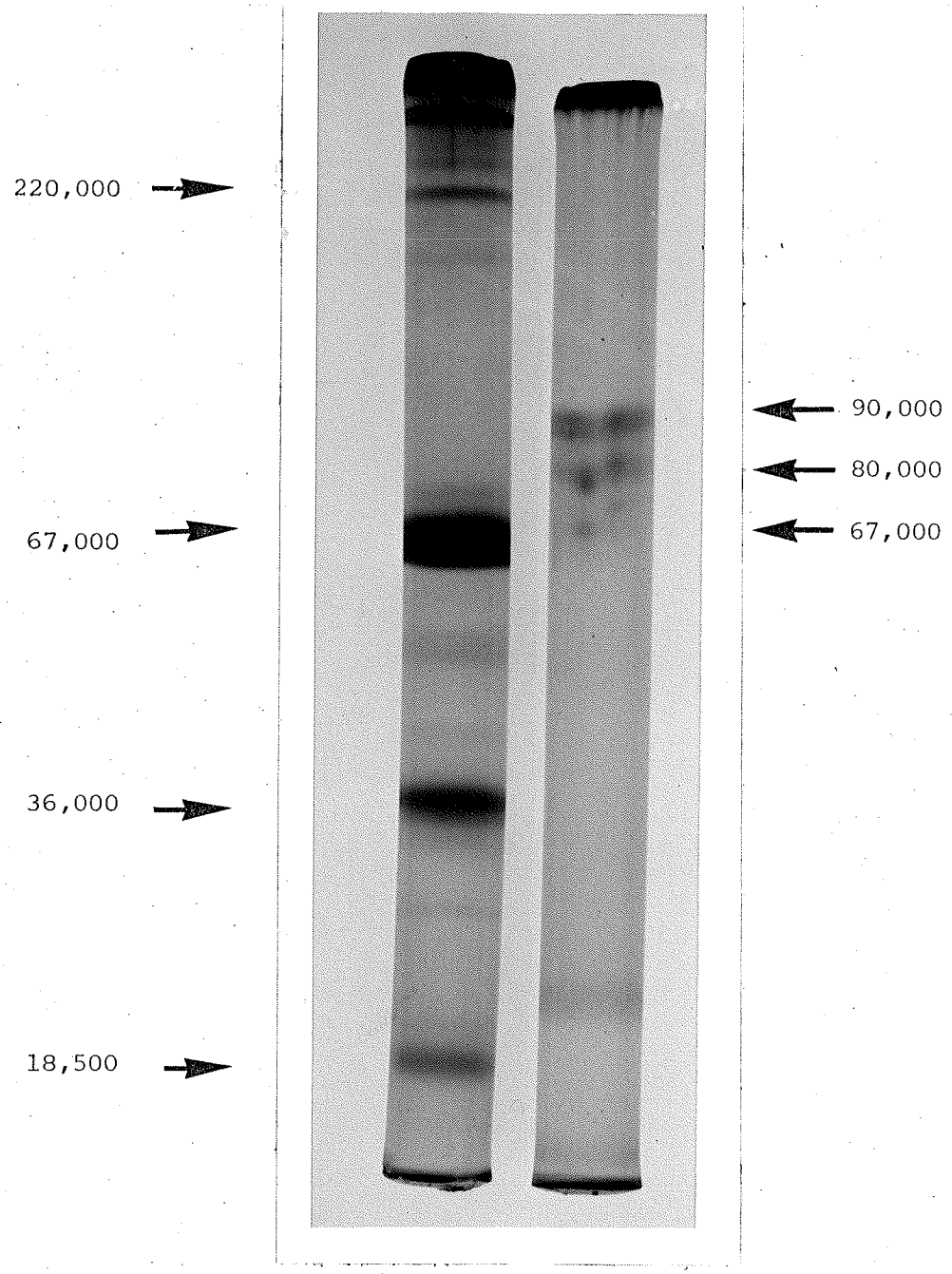

FIGURE 8. S.D.S. polyacrylamide gel electrophoresis of the ATPase. The protein band associated with the ATPase activity in Fig. 1 was treated with S.D.S. and electrophoresised under denaturing conditions in 10% polyacrylamide gels. The subunits of Mr= 90,000, 80,000 and 67,000 were observed.

FIGURE 9. Migration of the ATPase in different percentages of polyacrylamide gels. About 7 µg of purified protein was applied to (1) 5%, (2) 7.5%, (3) 10%, (4) 15% polyacrylamide.

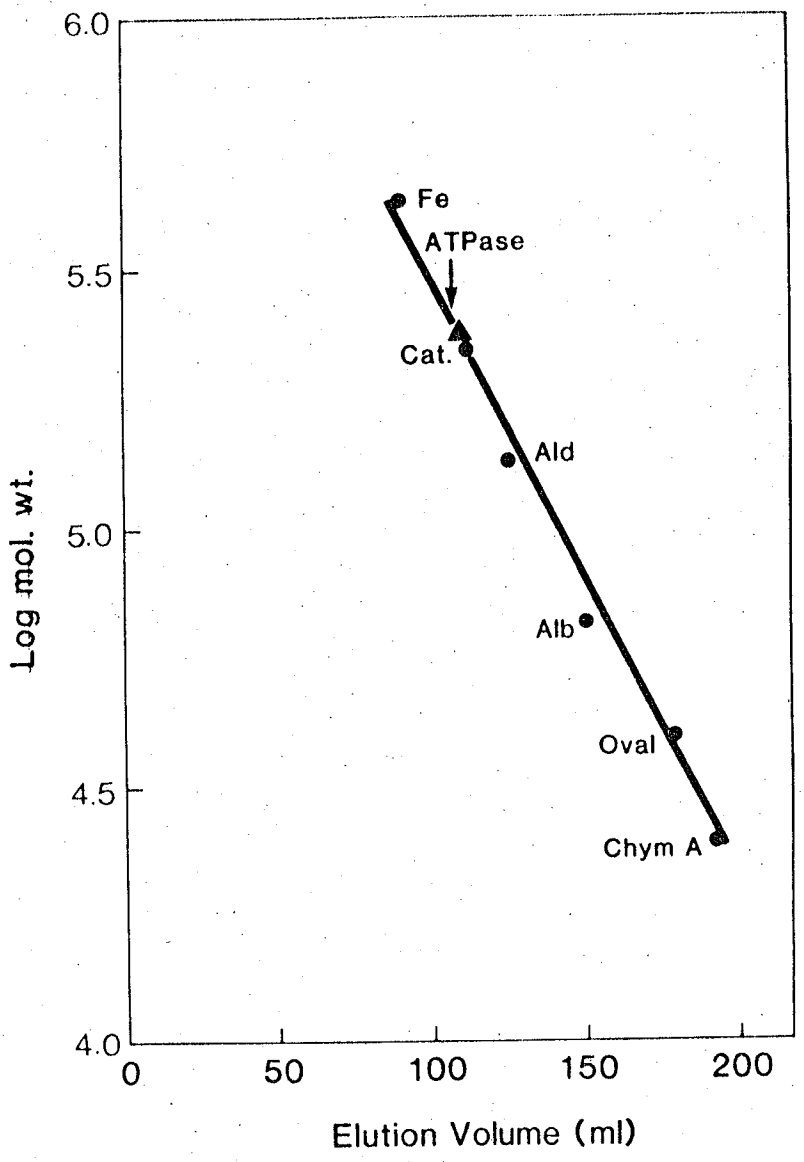

FIGURE 10. Estimation of the molecular weight of the ATPase on a Sephadex-G200 column. The elution volume of the ATPase was determined in relation to proteins of known molecular weight. About 1 mg of the enzyme protein was applied to the column and was eluted at a flow rate of 10 - 12 mls/hr.

Kinetic Properties - In one series of experiments, the enzyme was incubated in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+} and the hydrolysis was measured by employing different concentrations of ATP. Lineweaver-Burke analysis of the data indicated two different sites for Ca-ATP (Fig. 11A); a high affinity site with a K_m of 0.16 mM and a low affinity site with a K_m of 1 mM were observed. Similarly with Mg-ATP as substrate, a high affinity site with a K_m of 0.12 mM and a low affinity site with a K_m of 1 mM were observed (Fig. 11B). In another series of experiments the enzyme was incubated with 4 mM ATP and the hydrolysis measured by employing different concentrations of Ca^{2+} or Mg^{2+}. Lineweaver-Burke analysis of the data indicates a K_a value of 0.4 mM for Ca^{2+} (Fig. 12A) and a K_a of 0.2 mM for Mg^{2+} (Fig. 12B). The V_{max} values were 266 and 100 μmoles Pi/mg/hr for Ca^{2+} and Mg^{2+} respectively.

Ion and Substrate Specificity - In order to test the ion specificity of the ATPase, the enzyme was incubated with 4 mM ATP and the appropriate divalent cation at a concentration of 1.25 mM or 4 mM (Table VII). The ATPase was predominantly activated by Ca^{2+}, Mg^{2+} and Mn^{2+}. Sr^{2+}, Ba^{2+}, Ni^{2+} and Cu^{2+} were also able to activate the enzyme but to a lesser extent.

Substrate specificity of the ATPase was investigated by incubating the enzyme with 4 mM Ca^{2+} or 4 mM Mg^{2+} with the various nucleotides (Table VIII). The enzyme was able to utilize different nucleotides as a substrate, although the order of nucleotide hydrolysis in the presence of Ca^{2+} was different from that in the presence of Mg^{2+}.

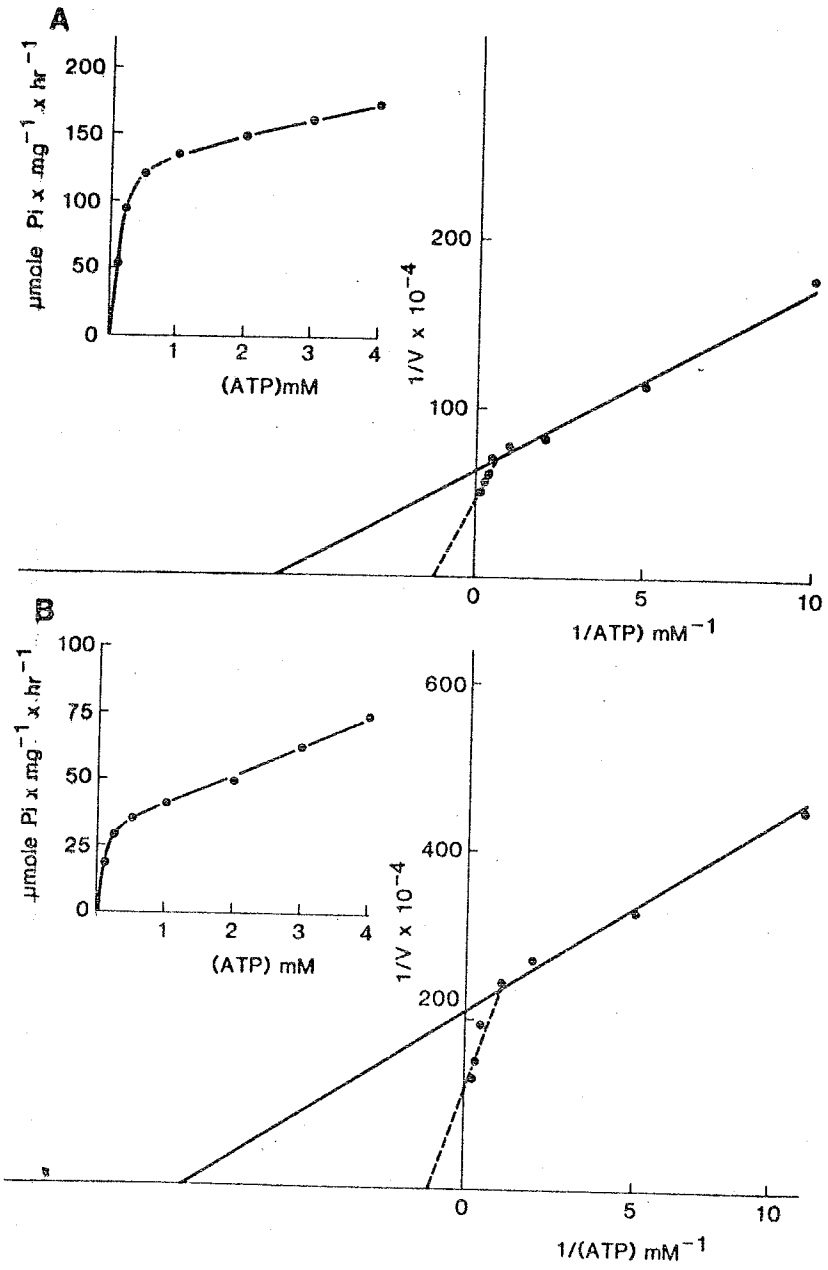

FIGURE 11. Effect of different concentrations of ATP on ATP hydrolysis by the partially purified ATPase due to the presence of (A) 4 mM Ca^{2+} or (B) 4 mM Mg^{2+}. The values are average of 3 experiments.

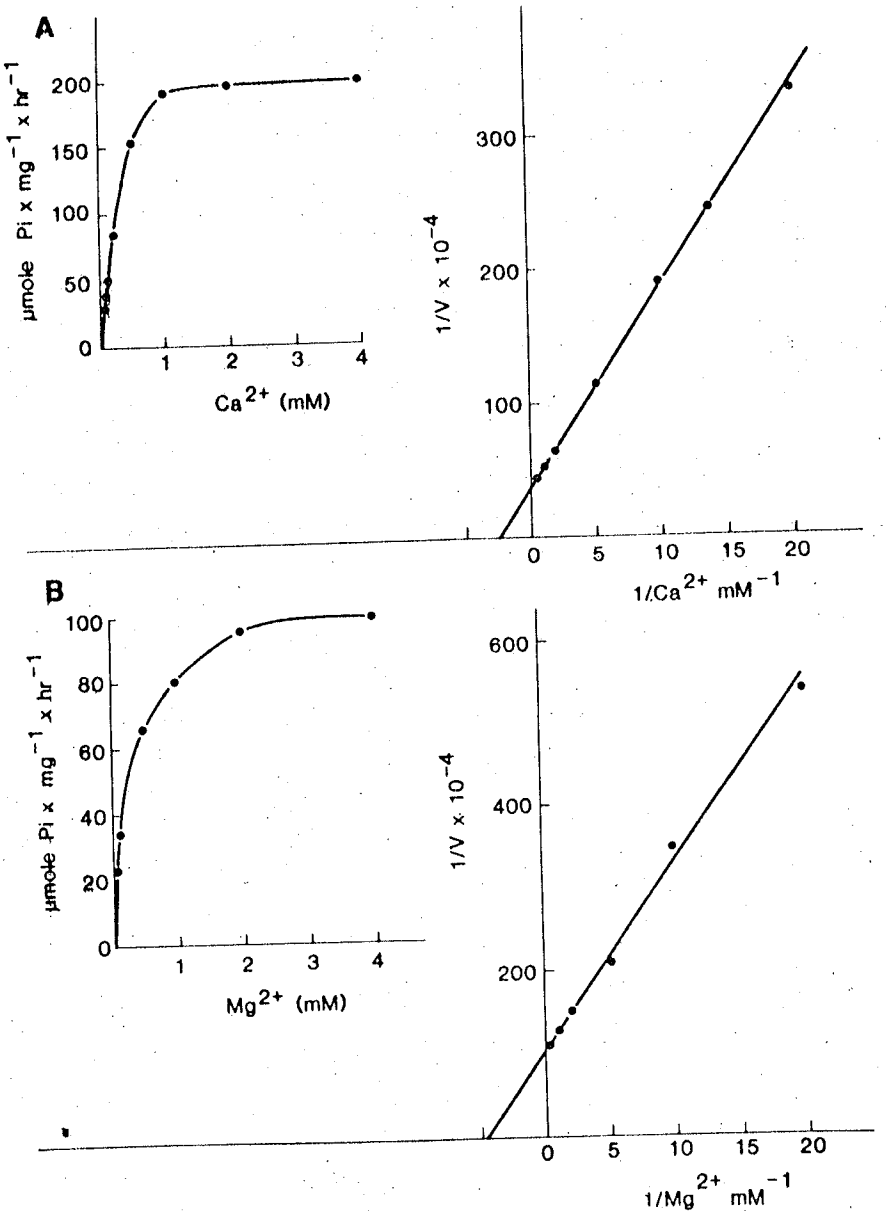

FIGURE 12. Effect of different concentrations of (A) Ca^{2+} or (B) Mg^{2+} on the ATP hydrolyzing ability of the partially purified ATPase. The incubation medium contained 50 mM Tris-HCl, pH 7.4, 1 mM EDTA and 4 mM ATP. Each value is an average of 3 different preparations.

TABLE VII. Ion Specifity of the Divalent Cation ATPase.

Cation	ATPase Activity (μmole Pi/mg/hr)	
	4 mM	1.25 mM
Ca^{2+}	209.2	168.2
Mg^{2+}	100.5	52.1
Mn^{2+}	76.2	31.3
Sr^{2+}	57.6	20.2
Ba^{2+}	14.6	3.6
Ni	14.6	2.8
Cu	14.6	3.1

Purified enzyme (5 μg) was incubated in the presence of 1.25 mM or 4 mM of different cations in a medium containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA and 4 mM ATP, and the reaction carried out as described in Methods. The results are typical of two different purified preparations. Each assay was performed in duplicate.

TABLE VIII. Substrate Specificity of Divalent Cation ATPase.

Nucleotide	4 mM Ca^{2+}	4 mM Mg^{2+}
ATP	191.4	85.4
CTP	127.6	19.6
GTP	113.5	70.3
ITP	123.2	56.2
UTP	120.1	27.02
ADP	20.6	15.4
AMP	4.1	5.1

Hydrolysis of each nucleotide is expressed as μmoles Pi/mg protein/hr. Nucleotide concentration used was 4 mM and the incubation medium contained 50 mM Tris-HCl, pH 7.5 and 1 mM EDTA. The results are a typical of three different partially purified preparations.

Effect of Divalent Cations - The effect of addition of various divalent cations, in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+} on the ATPase activity is shown in Table IX. The ATPase activity measured in the presence of Ca^{2+} was inhibited 50 to 75% by Mg^{2+}, Mn^{2+}, Ni^{2+} and Cu^{2+}. Ba^{2+} was essentially without effect on this ATPase activity whereas Sr^{2+} inhibited slightly. On the other hand, the ATPase activity measured in the presence of 4 mM Mg^{2+} was not as potently inhibited by any of the divalent cations except Ni^{2+}, which inhibited by about 50%.

Effect of Monovalent Cations - Table X shows that the monovalent cations Na^{+}, K^{+}, NH_4^{+} and Li^{2+} had no appreciable effect on the divalent cation dependent ATPase activity measured in the presence of Ca^{2+} or Mg^{2+}.

Effect of Inhibitors - As shown in Table XI, the enzyme was insensitive to the Na^{+} - K^{+} ATPase inhibitor, ouabain (123) as well as to the mitochondrial ATPase inhibitors, oligomycin and sodium azide. Verapamil and D-600, which are known Ca^{2+} "channel" blockers (124) were without effect on the ATPase activity. The enzyme was also found to be insensitive to Vanadate, which is known to be a potent inhibitor of different ATPases, including the sarcolemmal and the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+}) ATPase (80).

pH Optima - The enzyme was incubated in a medium at different pH and the ATPase activity determined (Fig. 13). Optimum pH for the ATPase activity was between 8.5 to 9.0, although there was no sharp pH optima.

TABLE IX. Effect of Divalent Cations on the Ca^{2+} and Mg^{2+} Dependent ATP Hydrolysing Ability of Divalent Cation Dependent ATPase.

Cation	Divalent Cation ATPase (μmoles Pi/mg/hr)	
	4 mM Ca^{2+}	4 mM Mg^{2+}
Control	178.4	81.6
Ca^{2+}	170.2	81.1
Mg^{2+}	77.8	78.3
Mn^{2+}	94.1	64.8
Sr^{2+}	142.7	68.1
Ba^{2+}	175.1	68.1
Ni^{2+}	40.5	40.6
Cu^{2+}	64.3	61.6

The experimental conditions were the same as those for Table VI. 4 mM of each divalent cation was added in the presence of 4 mM Ca^{2+} or Mg^{2+}. The results are average of three different partially purified enzyme preparations.

TABLE X. Effect of Monovalent Ions on Divalent Cation ATPase Activity.

	Divalent ATPase Activity (μmoles Pi/mg/hr)	
	4 mM Ca^{2+}	4 mM Mg^{2+}
Control	178.4	77.8
Na^{+} (100 mM)	168.1	72.9
K^{+} (100 mM)	165.6	71.4
NH_4^{+} (100 mM)	172.5	74.1
Li^{+} (100 mM)	167.5	71.2

The experimental conditions were the same as those for Table IV. The results are average of 3 different partially purified enzyme preparations.

TABLE XI. Effect of Various Inhibitors on Divalent Cation ATPase.

Inhibitor	Divalent ATPase Activity (μmoles Pi/mg/hr)	
	4 mM Ca^{2+}	4 mM Mg^{2+}
Control	187.4	82.3
Ouabain (2 mM)	180.5	75.7
Verapamil (5 μM)	182.7	76.8
D-600 (5 μM)	182.7	70.5
Vanadium (100 μM)	186.9	74.1
Oligomycin (50 μM)	182.7	73.5
Sodium azide (1 mM)	179.7	76.7

The experimental conditions were similar to those described in Table IV, except that the indicated concentration of the inhibitor was included. The results are average of three different purified enzyme preparations.

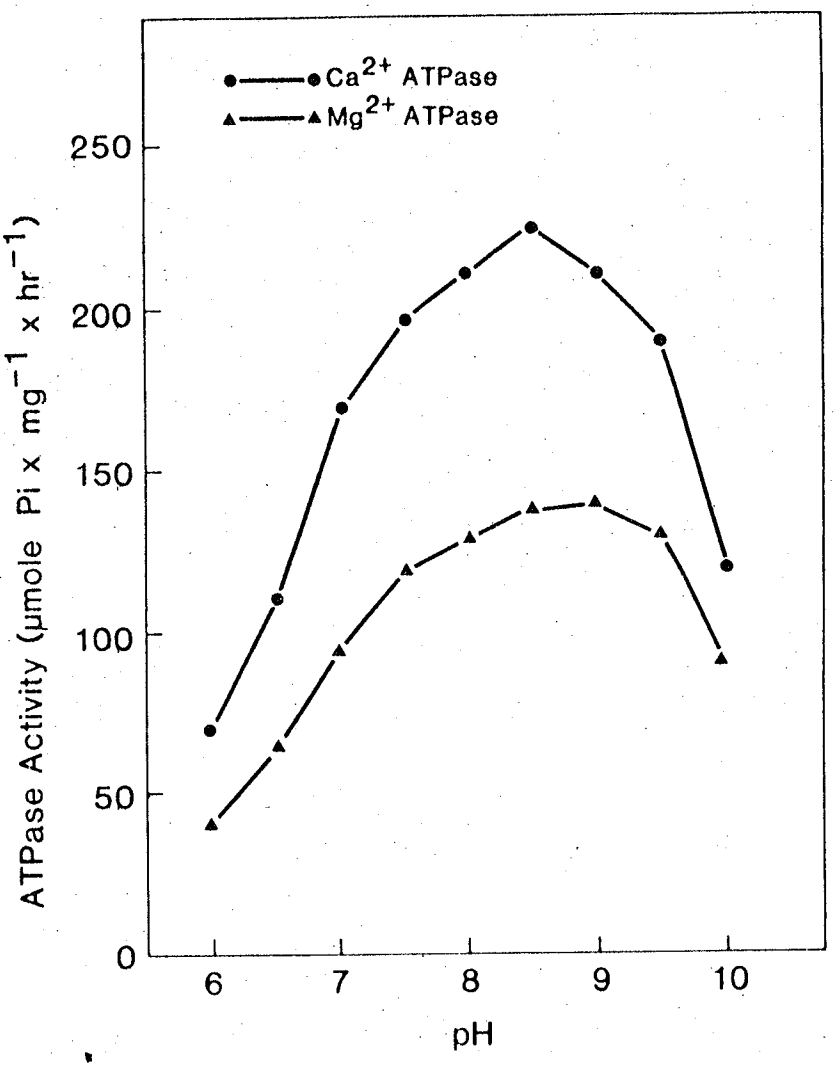

FIGURE 13. pH profile of the ATP hydrolyzing activities of the ATPase in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+}. Each value is an average of 3 different preparations.

Effect of Calmodulin - Calmodulin is known to activate calcium stimulated enzymes (125). Calmodulin stimulation of the (Ca^{2+} + Mg^{2+}) ATPase of heart sarcolemma as well as the erythrocyte membrane was associated with an increase in V_{max} and a decrease in K_a for Ca^{2+} (81). The presence of calmodulin in heart sarcolemma was recently demonstrated (80). Table XII shows that calmodulin did not stimulate the purified divalent cation dependent ATPase at any of the concentrations tested. In fact a slight inhibition of the ATPase activity was observed. The calmodulin-antagonist, trifluoroperazine, also inhibited the ATPase activity in the presence of Ca^{2+} or Mg^{2+}. Trifloroperazine and calmodulin together also inhibited the ATPase activity. Calmodulin failed to induce any appreciable ^{32}P incorporation in the ATPase enzyme (Table XIII). The purified enzyme did not bind to the calmodulin affinity column suggesting that there is no interaction between calmodulin and the ATPase (Fig. 14).

Effect of cAMP - Protein Kinase - To test if the ATPase may be regulated by the process of phosphorylation, the ability of cAMP dependent catalytic subunit of protein kinase to phosphorylate the enzyme was investigated. Table XIII shows that cAMP did not result in any significant ^{32}P incorporation; however, about 0.46 m moles of ^{32}P were incorporated per mole of enzyme in the presence of the catalytic subunit of protein kinase. The time dependency of catalytic subunit induced ^{32}P incorporation is shown in Table XV. Maximum ^{32}P incorporation was achieved within 12 min of incubation with the

TABLE XII. Effect of Calmodulin on Purified Divalent Cation Dependent ATPase.

	Ca^{2+}		Mg^{2+}	
	1.25 mM	4 mM	1.25 mM	4 mM
Control	280	324	100.6	205.3
Calmodulin				
1 µg	281.1	315.9	101.1	202.1
7 µg	278.5	292	98.4	209.5
15 µg	262.3	286.1	97.8	203.5
TFP (10 µM)	229.6	268.9	82.5	174.5
Calmodulin (7 µg)				
+ TFP (10 M)	232.4	270.6	81.4	171.3

ATPase activity (µmoles Pi/mg/hr) was determined in the presence of 1.25 mM or 4 mM of the divalent cation concentration, with or without calmodulin and TFP in the same incubation medium as in Table IV. The results are[a] typical of two purified enzyme preparations.

TABLE XIII. Effect of cAMP - Dependent and Calcium-Calmodulin Dependent Phosphorylation of the Divalent Cation ATPase

Additions	Divalent Cation ATPase µmole Pi/mg/hr		^{32}P Incorporation m moles/mole enzyme
	4 mM Ca^{2+}	4 mM Mg^{2+}	
Control	184.8	142.6	- -
cAMP (5 µM)	155.6	128.3	0.07
Calmodulin (7 µg)	171.4	141.3	0.06
Catalytic subunit of Pk (150 units)	148.7	103.6	0.46

5 - 10 µg of purified enzyme was preincubated with cAMP, or calmodulin or the catalytic subunit for 30 min and the ATPase activity and ^{32}P incorporation determined as described in Methods. The results are average of two different purified preparations.

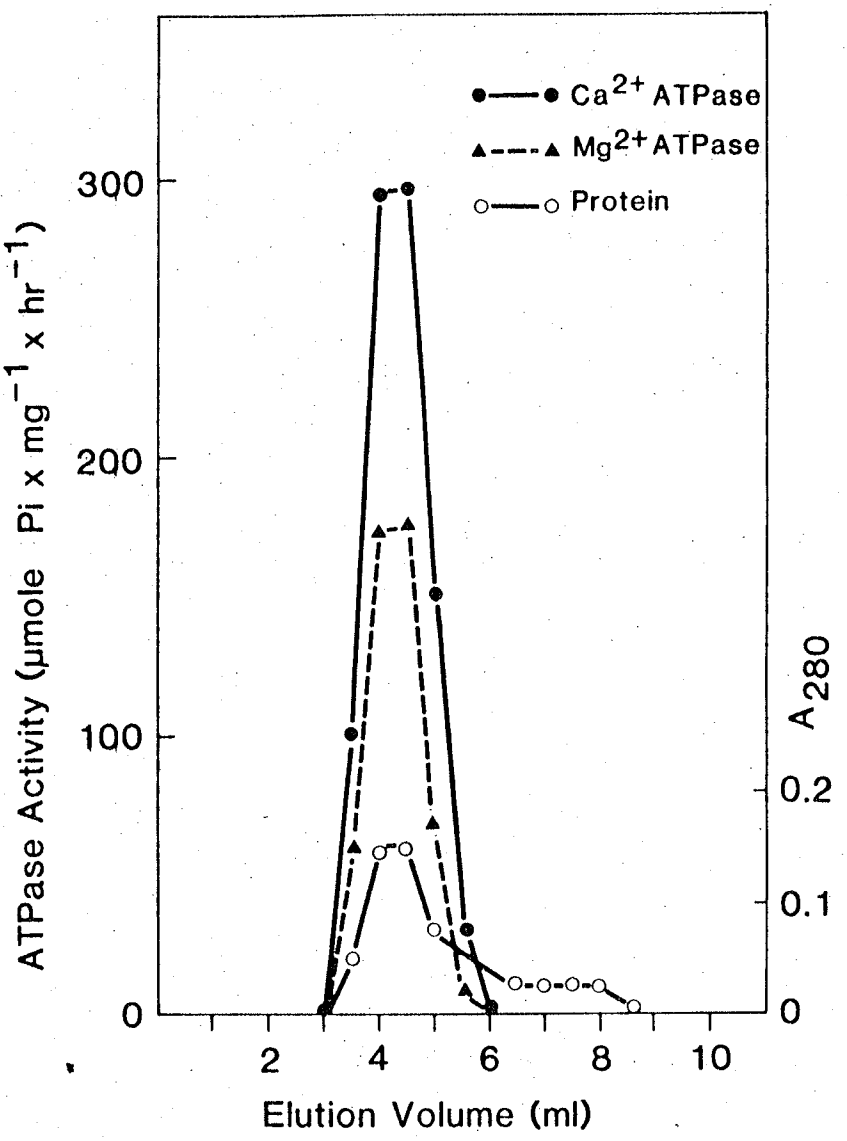

FIGURE 14. Elution profile of the ATPase on a calmodulin affinity column. About 150 µg of purified enzyme was applied to the column which was eluted as described in "Methods".

TABLE XV. Time Dependency of Catalytic Subunit Induced Phosphorylation and ATPase Activity

Time of Incubation (min)	^{32}P Incorporation mmoles/mole enzyme	ATPase Activity	
		4 mM Ca^{2+}	4 mM Mg^{2+}
0	- -	184.8	142.6
1	0.19	171.3	132.4
5	0.37	151.6	110.5
12	0.46	146.9	105.3
20	0.45	147.3	104.1
30	0.46	140.2	100.9

5 - 10 µg of purified enzyme was incubated with the catalytic subunit of Pk and the ^{32}P incorporation and ATPase activity measured at various time intervals. The results are average of two different preparations.

catalytic subunit, and the ATPase activity in the presence of Ca^{2+} or Mg^{2+} was found to be inhibited about 25 to 30% (Table XV).

V. DISCUSSION

Heart sarcolemma exhibits a divalent cation ATPase activity which is dependent on Ca^{2+} or Mg^{2+}. Activation of this membrane bound enzyme in rat heart in the presence of Mg^{2+} is about 70% of that observed with Ca^{2+}. The ATPase activity in dog heart sarcolemma in the presence of Mg^{2+} is about 150% of that in the presence of Ca^{2+}. It is therefore not known whether the hydrolysis of ATP in the presence of Ca^{2+} or Mg^{2+} is due to a single enzyme or two different enzymes in the sarcolemma. Treatment of the rat heart sarcolemma with deoxycholate has been shown to decrease Mg^{2+} ATPase activity without affecting the Ca^{2+} ATPase activity (106). Furthermore, the sensitivities of these enzyme activities to various inhibitors were found to be different from each other (126). Since the ATPase purified from the rat heart membrane is activated by both Ca^{2+} and Mg^{2+}, it can be stated that sarcolemma contains an enzyme which has a specificity for the divalent cations. In dog heart, the activity of the purified ATPase in the presence of Ca^{2+} was about 25% of that in the presence of Mg^{2+}; Ca^{2+} ATPase activity in this preparation decreased markedly without affecting the Mg^{2+} ATPase activity upon sonication at 37°C. On the other hand, the activity of rat heart purified ATPase in the presence of Ca^{2+} was markedly higher than that in the presence of Mg^{2+}. In fact under different experimental conditions, Mg^{2+} ATPase of the rat heart enzyme was much more labile in comparison to Ca^{2+}

ATPase. Thus it appears that the ATP hydrolyzing activity of the heart sarcolemma in the presence of different cations may be due to the same enzyme but its sensitivity to cations may vary in different animal species as well as under different experimental conditions. The possible explanation of this phenomenon may depend upon the presence of a factor, which may confer the sensitivity of the enzyme complex to either Mg^{2+} or Ca^{2+}. Such a factor has been shown to be present in the cell membrane and was reported to be of lipid nature (128). Furthermore, actin is well known to confer Mg^{2+} ATPase activity to myosin, which is a Ca^{2+} ATPase enzyme.

The ATPase purified in this study is unique in a number of ways when compared with other ATPases. For example, this ATPase does not exhibit any Ca^{2+} stimulated Mg^{2+} dependent ATPase activity and is thus different from the (Ca^{2+} + Mg^{2+}) ATPase of the sarcoplasmic reticulum and plasma membrane (3, 80). While the monovalent cations, Na^{+} and K^{+} are activators of (Ca^{2+} + Mg^{2+}) ATPase (127), the ATPase purified here was shown to be insensitive to Na^{+} and K^{+}. Furthermore, the (Ca^{2+} + Mg^{2+}) ATPase was activated fully by 5 - 10 μM Ca^{2+} (K_a = 2 μM); concentrations of Ca^{2+} higher than 100 μM being inhibitory (3). On the other hand, the ATPase purified from sarcolemma was activated fully by 2 - 4 mM Ca^{2+} (K_a = 0.40 mM). In contrast to the (Ca^{2+} + Mg^{2+}) ATPase of sarcolemma (80), the purified ATPase was neither stimulated by calmodulin nor inhibited by vanadate. The molecular weight of the (Ca^{2+} + Mg^{2+}) ATPase of sarcolemma has been estimated

to be about 150,000 (80) whereas that of the sarcoplasmic reticular enzyme is about 100,000 (2,3). The molecular weight of the sarcolemmal ATPase purified here has been found to be about 240,000. These observations distinguish the divalent cation dependent ATPase from the (Ca^{2+} + Mg^{2+}) ATPase. In view of the differences in properties of these two enzymes and mM concentrations of Ca^{2+} required for the activity, it is suggested that the function of the divalent cation dependent ATPase is not one of Ca^{2+} extrusion as is thought to be the case for the (Ca^{2+} + Mg^{2+}) ATPase. On the other hand, in view of the cytosolic concentrations of Mg^{2+} under normal situation as well as the kinetic properties of the purified enzyme, it is likely that the divalent cation dependent ATPase may serve as a "Mg^{2+} pump".

The lack of response of the ATPase to ouabain, and oligomycin as well as sodium azide distinguish this enzyme from the sarcolemmal Na^{+} + K^{+} ATPase and mitochondrial ATPase, respectively. Further distinction between the divalent cation ATPase and the Na^{+} + K^{+} ATPase was indicated by the lack of reactivity of antiserum raised against the Na^{+} + K^{+} ATPase with the divalent cation ATPase. The purified ATPase also differs markedly from myosin ATPase since myosin ATPase is activated fully by 10 mM Ca^{2+} (K_a = 2 mM), with a V_{max} of about 60 μmoles Pi/mg/hr (129); these kinetic characteristics being markedly different from the divalent cation ATPase. Furthermore, the divalent cation dependent ATPase can only be solubilized from the sarcolemma by the use of trypsin and detergents, whereas myosin can be

extracted in high salt solutions. Contractile proteins, however, have been shown to be present in plasma membrane preparations from a variety of tissues (130), and it may be that the divalent cation dependent ATPase may be related to such a protein situated in the sarcolemma. But no reactivity of the antiserum against myosin was observed with the divalent cation dependent ATPase, and neither was any K-EDTA stimulated ATPase activity associated with the purified ATPase. These observations distinguish the divalent cation dependent ATPase from myosin.

The sarcolemmal membrane fraction obtained from rat heart, contains an ATPase activity of about 30 μmoles Pi/hr per mg of membrane protein. The specific activity of the pure enzyme is about 340 μmoles Pi/hr per mg of protein. Thus the ATPase accounts for about 8% of the plasma membrane protein. The purification procedure described here yields about 4 μg of pure enzyme per g of heart tissue. Although the divalent cation dependent ATPase activity has been reported to be present in plasma membrane preparations from a variety of mammalian tissues, this is the first report describing the solubilization and purification of the membrane bound enzyme. The results indicate that the native enzyme has a molecular weight of about 240,000 and consists of three major subunits with molecular weight of about 90,000, 70,000 and 67,000.

Work with myocytes indicate that the divalent cation dependent ATPase is an ecto-enzyme of the heart cell membrane (104). On the

other hand, sarcolemmal membrane preparations with predominantly "inside out" orientation also exhibited divalent cation dependent ATPase activity (131) suggesting that the enzyme has a catalytic site on the cytoplasmic face of the plasma membrane also. Since the use of the detergent TX-100 was necessary to solubilize the ATPase, strong lipid association of the protein in the membrane is indicated and it is conceivable that the enzyme may be a transmembranal protein. Trypsin treatment of sarcolemma appears to be a prerequisite inorder to solubilize the divalent cation dependent ATPase with TX-100. Trypsin solubilizes a protein of molecular weight 67,000 that exhibits Ca^{2+} - dependent ATPase activity (83). It should be pointed out that unlike the divalent cation dependent ATPase, the Ca^{2+} - dependent ATPase showed a negligible activity in the presence of Mg^{2+} (83). The fact that the Ca^{2+} - dependent ATPase can be solubilized without the use of detergents, indicates that this protein exists in a hydrophillic environment as compared with the divalent cation dependent ATPase, which appears to be an intrinsic protein situated in the hydrophobic lipid environment. Furthermore, unlike the divalent cation ATPase, the Ca^{2+} - dependent ATPase exhibits Ca^{2+} - Na^{+} antagonistic sites and is markedly inhibited by Mg^{2+} (83). We have also shown that Ca^{2+} - dependent ATPase is an ecto-enzyme of the heart cell membrane, and can be solubilized from cardiac myocytes with trypsin treatment. Although, various characteristics of the Ca^{2+} - dependent ATPase suggest that it may be a distinct enzyme from the

divalent cation dependent ATPase, the possibility that Ca^{2+} - dependent ATPase is a tryptic fragment of a larger molecule can not be ruled out, at present. Trypsin treatment of the (Ca^{2+} + Mg^{2+}) ATPase of sarcoplasmic reticulum was shown to result in degradation of the 102,000-dalton enzyme to two fragments of 55,000 and 45,000 daltons with subsequent appearance of fragments of 30,000 and 20,000 daltons (132). Although no ATPase activity was detected in any of the purified fragments, the 30,000-dalton fragment was found to contain the site of phosphorylation and the 20,000-dalton fragment may serve as the Ca^{2+} ionophore (133). The tryptic fragment of the sarcolemmal ATPase not only have different physical and chemical characteristics, but may also have individual functional roles, which together constitute the characteristics and function of the native holoenzyme.

If Ca^{2+} - dependent ATPase is a fragment of the divalent cation dependent ATPase, it can be envisioned to be located on the extracellular face of the plasma membrane, with the more hydrophobic portion of the enzyme embedded in the lipid bilayer. The divalent cation dependent ATPase may be a oligomeric protein which spans the lipid bilayer. In this regard, oligomeric proteins, which traverse the membrane, appear well suited to the function of translocating ions and nutrients across the cell membrane (134). A clear example of transmembrane arrangement of a multisubunit membrane protein is the (Na^+ + K^+) - ATPase which serves as a "Na^+ pump". The sites responsible for ATP hydrolysis in both subunits must be directed towards the

cytosolic face and electron microscopy shows that ($Na^+ + K^+$) ATPase bulges only towards the inner side of the cell. Each large subunit of the ($Na^+ + K^+$) ATPase binds a minimum of one 53,000 molecular weight glycoprotein, which faces the extracellular space (135). A proteolipid of molecular weight 12,000 is thought to form the ion channel through the cell membrane (45). Thus the divalent cation dependent ATPase, as a integral protein of the sarcolemmal membrane, consisting of subunits is well suited to have carrier functions.

The sarcolemmal divalent cation dependent ATPase has been suggested to serve as a biochemical correlate of the Ca^{2+} channel involved in the entry of Ca^{2+} into the cardiac cell (1). The problem of finding physical counterparts to electrophysiological data is that of how to relate an alteration in electric field to a movement of ions- in biochemical terms a conformational change - which brings about the opening of an ion channel. Furthermore, electrophysiological studies leave fundamental questions regarding the physical and chemical nature of the structures of channels unanswered. Biochemical studies of the sodium channel has resulted in the isolation of a protein of molecular weight 250,000, which consists of four subunits of which a 76,000 molecular weight component, associated with a 56,000 molecular weight component is the target for a photoaffinity labelled scorpion toxin (136). Thus the subunit structure and the transmembranal nature of the divalent cation dependent ATPase could possibly be conceived to be involved in the Ca^{2+} channel activity. The hydrolysis of ATP may

be envisioned to "open or close" the calcium channel (i.e. a conformational change) and this may be the function of the ecto-ATPase. However, divalent cation conductivity measurements of the translocation of Ca^{2+} need to be carried out for any meaningful conclusion. The lack of effect of the Ca^{2+} antagonists, verapamil and D-600, on the ATPase enzyme may be due to a change in the conformation of the purified protein. In this regard, verapamil has been shown to inhibit the sarcolemmal membrane bound ATPase activity. The cations, Ni^{2+}, Co^{2+} and Mn^{2+}, which are known to block Ca^{2+} entry into the cell, were found to be potent inhibitors of the divalent cation dependent ATPase. In this regard, divalent cation ecto-ATPase has also been implicated to be involved in the process of Ca^{2+} entry in other types of cells (102). Although the activity of the sarcolemmal bound ATPase has been shown to increase in the presence of cAMP dependent protein kinase (110), the activity of the purified ATPase was slightly inhibited as a result of phosphorylation. This may be due to some changes in the conformation of the enzyme during purification and the removal of some component from the enzyme complex.

The low affinity of the ATPase for ATP (1 mM) indicates that this enzyme may be a major source of energy utilization in the heart cell. The energy may be utilized not only to open and close Ca^{2+} channels, but also to maintain the structure and dynamics of the cell membrane. In this regard, the sarcolemma has been shown to contract in the presence of ATP (137) and the divalent cation dependent ATPase

may be involved in this process. Furthermore, this enzyme could also serve to synthesize ATP in the sarcolemmal membrane in a manner similar to that proposed for the sarcoplasmic reticular Ca^{2+} - stimulated Mg^{2+} dependent ATPase or mitochondrial Mg^{2+} ATPase (3, 4). It is also possible that the ecto-ATPase, by generating protons during the hydrolysis of ATP, may participate in the transport of different substrates and amino acids across the cell membrane. In view of such suggestions, it can be appreciated that extensive work needs to be carried out to discover the functions of the divalent cation dependent ATPase.

VI. REFERENCES

1. Dhalla, N.S., Ziegelhoffer, A. and Harrow, J.A.C. (1977). Can. J. Physiol. Pharmacol. 55, 1211 - 1234.
2. MacLennan, D.H. and Holland, P.C. (1975). Ann. Rev. Biophys. Bioeng. 4, 377 - 404.
3. Tada, M., Mamamoto, T., Tonomura, y. (1978). Physiol. Rev. 58, 1 - 78.
4. Penefsky, H.S. (1979). Adv. Enzymol. 49, 223 - 280.
5. Carafoli, E. and Crompton, M. (1978). In Current Topics in Membranes and Transport. 10, 151 - 198.
6. Dhalla, N.S., McNamara, D.B. and Anand, M.B. (1976). In Recent Advances in Studies on Cardiac Structure and Metabolism. 5, 1 - 19.
7. Reuter, H. (1979). Ann. Rev. Physiol. 41, 413 - 424.
8. McNutt, N.S. and Fawcett, D.W. (1974). In Mammalian Myocardium, edited by Langer, G.A. and Brady, A.J. pp. 1 - 50.
9. McNutt, N.S. (1975). Circ. Res. 37, 1 - 13.
10. Parsons, D.F. and Subjeck, J.R. (1972). Biochim. Biophys. Acta 265, 85 - 92.
11. Howse, H.D., Ferrans, V.J. and Hibbs, R.G. (1970). J. Mol. Cell. Cardiol. 1, 157 - 167.
12. Frank, J.S., Langer, G.A., Nudd, L.M. and Seraydarian, K. (1977). Circ. Res. 41, 274 - 283
13. Rich, T.L. and Langer, F.A. (1975). J. Mol. Cell. Cardiol. 7, 747 - 760.

14. Williamson, J.R., Woodrow, M.L. and Scarpa, A. (1975). In Recent Advances in STudies in Cardiac Structure and Metabolism. Edited by Fleckenstein, A. and Dhalla, N.S. 5, 61. University Park Press, Baltimore.

15. Limas, C.J. (1977). Arch. Biochem. Biophys. 179, 302 - 312.

16. Langer, G. (1976). Fed. Proc. 35, 1274 - 1278.

17. Singer, S.J. and Nicolson, G.L. (1972). Science 175, 720 - 731.

18. Chapman, D., Gomez-Fernandez, J.C. and Goni, F.M. (1982). TIBS 7, 67 - 70.

19. Skou, J.C. (1957). Biochim. Biophys. Acta 23, 394 - 401.

20. Bonting, S.L. (1970). Membranes and Ion Transport, edited by Bittar, E.E., London: Wiley Interscience, Vol. 1, pp. 257 - 363.

21. Akera, T., Larsen, F.S. and Brody, T.M. (1969). J. Pharmacol. Exp. Ther. 170, 17 - 26.

22. Sen, A.K. and Post, R.L. (1964). J. Biol. Chem. 239, 345 - 352.

23. Baker, P.F., Blaustein, M.P., Keynes, R.D., Manil, J., Shaw, T.I. and Steinhardt, R.A. (1969). J. Physiol. London 200, 459 - 496.

24. Jorgensen, P.L. (1974). Biochim. Biophys. Acta 356, 53 - 67.

25. Hokin, L.E., Dahl, J.L., Deupree, J.D., Dixon, J.F., Hackney, J.F. and Perdue, J.F. (1973). J. Biol. Chem. 248, 2593 - 2605.

26. Dixon, J.F. and Hokin, L.E. (1974). Arch. Biochim. Biophys. 163, 749 - 758.

27. Hopkins, B.E., Wagner, J.,Jr. and Smith, T.W. (1976). J. Biol. Chem. 251, 4365 - 4371.

28. Uesugi, S., Dulak, N.C., Dixon, J.E., Hexum, T.D., Dahl, J.L., Perdue, J.F. and Hokin, L.E. (1971). J. Biol. Chem. 246, 531 - 543.

29. Pitts, B.J.R. and Schwartz, A. (1975). Biochim. Biophys. Acta 401, 184 - 195.

30. Lane, L.K., Copenhaver, J.J., Jr., Lindenmayer, G.E. and Schwartz, A. (1973). J. Biol. Chem. 248, 7197 - 7200.

31. Lee, H.C., Breitbart, H., Berman, M. and Forte, J.G. (1979). Biochem. Biophys. Acta 553, 107 - 131.

32. Peterson, G.L., and Hokin, L.E. (1981). J. Biol. Chem. 256, 3751 - 3761.

33. Haley, B.E. and Hoffman, J.F. (1974). Proc. Natl. Acad. Sci. U.S.A. 71, 3367 - 3371.

34. Hart, W.M. and Titus, E.O. (1973). J. Biol. Chem. 248, 4674 - 4681.

35. Alexander, A. (1974). FEBS Letts. 45, 150 - 154.

36. Jorgensen, P.L. and Petersen, J. (1977). Biochim. Biophys. Acta 466, 97 - 108.

37. Perrone, J.R., Hackney, J.F., Dixon, J.F. and Hokin, L.E. (1975). J. Biol. Chem. 250, 4178 - 4184.

38. Shamoo, A.E. and Myers, H. (1974). J. membr. Biol. 19, 163 - 178.

39. Rhee, H.M. and Hokin, L.E. (1975). Biochem. Biophys. Res. Commun. 63, 1139 - 1145.

40. Churchill, L. and Hokin, L.E. (1976). Biochim. Biophys. Acta 434, 258 - 264.

41. Reeves, A. S., Collins, J.H. and Schwartz, A. (1980). Biochem. Biophys. Res. Comm. 95, 1591 - 1598.

42. Collins, J.H., Forbush, B., Lane, L.K., Ling, E., Schwartz, A. and Zot, A. (1982). Biochim. Biophys. Acta 686, 7 - 12.

43. Forbush, B., Kaplan, J.H. and Hoffman, J.F. (1978). Biochemistry 17, 3667 - 3676.

44. Folch, J. and Lees, M. (1951). J. Biol. Chem. 191, 807 - 817.

45. Racker, E. (1976). Trends Biochem. Sci. 1, 244.

46. Rogers, T.B. and Lazdunski, M. (1979). FEBS Lett. 98, 373 - 376.

47. Dowd, F.J., Pitts, B.J.R. and Schwartz, A. (1976). Arch. Biochem. Biophys. 175, 321 - 331.

48. Stekhoven, F.S. and Bonting, S.L. (1981). Physiol. Rev. 61, 1 - 76.

49. Tanaka, R. and Abood, L.G. (1964). Arch. Biochem. Biophys. 108, 47 - 52.

50. Hilden, S. and Hokin, L.E. (1976). Biochem. Biophys. Res. Commun. 69, 521 - 527.

51. Tsien, R.W. (1977). Adv. Cyclic Nucleotide Res. 8, 363 - 420.

52. Tada, M., Kirchberger, M.A., Repke, D.I. and Katz, A.M. (1974). J. Biol. Chem. 249, 6174 - 6180.

53. Nayler, W.G. and Berry, D. (1975). J. Mol. Cell. Cardiol. 7, 387 - 395.

54. Sulakhe, P.V., Leung, N.L. and St. Louis, P.J. (1976). Can. J. Biochem. 54, 438 - 445.

55. Insel, P.A., Maguires, M.E., Gilman, A.G., Bourne, H.R., Coffino, P., Melon, K.L. (1976). Mol. Pharmacol. 12, 1062 - 1069.

56. Vauguelin, G., Geynet, P., Hanounre, J. and Strosberg, A.D. (1977). Proc. Natl. Acad. Sci. U.S.A. 74, 3710 - 3714.

57. Vanguelin, G., Geynet, P., Honounre, J. and Strosberg, A.D. (1979). Eur. J. Biochem. 98, 543 - 556.

58. Atlas, D. and Levitzki, A. (1978). Nature 272, 370 - 371.

59. Caron, M.D. and Lefkowitz, R.J. (1978). J. Biol. Chem. 251, 2374 - 2384.

60. Orly, J. and Schraun, C. (1976). Proc. Natl. Acad. Sci. U.S.A. 73, 4410 - 4414.

61. Limbird, L.E. and Lefkowitz, R.J. (1977). J. Biol. Chem. 252, 799 - 802.

62. Homay, C., Wrenn, S. and Haber, E. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 59 - 63.

63. Neer, E.J. (1974). J. Biol. Chem. 249, 6527 - 6531.

64. Haga, T., Haga, K., Gilman, A.G. (1977). J. Biol. Chem. 252, 5776 - 5782.

65. Stengel, D., Hanounce, J. (1980). Eur. J. Biochem. 102, 21 - 34.

66. Pfeuffer, T. (1977). J. Biol. Chem. 252, 7224 - 7234.

67. Gill, D.M. and Meren, R. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 3050 - 3054.

68. Cassel, D., Pfeuffer, T. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 2669 - 2673.

69. Burger, R. and Lowenstein, J.M. (1970). J. Biol. Chem. 245, 6274 - 6280.

70. Frick, G.P. and Lowenstein, J.M. (1978). J. Biol. Chem. 253, 1240 - 1244.

71. Schultz, V. and Lowenstein, J.M. (1978). J. Biol. Chem. 253, 1938 - 1943.

72. Berne, R.M. and Rubio, R. (1979). Handb. Physiol. Sect. 2: Cardiovasc, Syst. 1, 873 - 952.

73. Evans, W.H. and Gurd, J.W. (1973). Biochem. J. 133, 189 - 199.

74. Basmann, H.B. and Pike, G.Z. (1971). Biochim. Biophys. Acta 227, 402 - 412.

75. Nakamura, S. (1976). Biochim. Biophys. Acta 426, 339 - 347.

76. Dornand, J., Bonnafous, J.C. and Mani, J.C. (1978). Eur. J. Biochem. 87, 459 - 465.

77. Naito, Y. and Lowenstein, J.M. (1981). Biochemistry 20, 5188 - 5194.

78. Sulakhe, P.V. and St. Louis, P.J. (1980). Prog. Biophys. Molec. Biol. 35, 135 - 195.

79. Niggli, V., Adunyah, E.S., Penniston, J.T. and Carafoli, E. (1981). J. Biol. Chem. 256, 395 - 401.

80. Caroni, P. and Carafoli, E. (1981). J. Biol. Chem. 256, 3263 - 3270.

81. Tuana, B.S., Dzurba, A., Panagia, V. and Dhalla, N.S. (1981). Biochem. Biophys. Res. Commun. 100, 1245 - 1250.

82. Caroni, P. and Carafoli, E. (1981). J. Biol. Chem. 256, 9371 - 9373.

83. Tuana, B.S. and Dhalla, N.S. (1982). Submitted for publication.

84. Dhalla, N.S., Anand-Srivastava, M.B., Tuana, B.S. and Khandelwal, R.L. (1981). J. Molec. Cell. Cardiol. 13, 413 - 423.

85. Anand, M.B., Chauhan, M.S. and Dhalla, N.S. (1977). J. Biochem. 82, 1731 - 1739.

86. Sulakhe, P.V., Drummond, G.I. and Ng, D.C. (1973). J. Biol. Chem. 248, 4158 - 4162.

87. Chambaut, A.M., Leroy-Pecker, F., Feldmann, G. and Hanoune, B.J. Jr. (1974). J. Gen. Physiol. 64, 104 - 126.

88. Vandermeers, A., Piret, M.C., Hepburn, A., Rathe, J. and Christophe, J. (1982). J. Biochem. 121, 295 - 299.

89. Majumder, G.C. and Biswas, R. (1979). Biochem. J. 183, 737 - 743.

90. Buckland, R.M., Radda, G.K. and Wakefield, L.M. (1979). FEBS Letts. 103, 323 - 327.

91. Russo, J. and Wells, P. (1977). J. Histochem. Cytochem. 25, 135 - 148.

92. Khan, M.S. and Ochs, S. (1974). Brain Res. 81, 413 - 426.

93. Trams, E.G., Lautev, C.L. and Salem, N. (1981). Comp. Biochem. Physiol. 69, 195 - 199.

94. Baskin, G.S. and Langdon, R.G. (1981). J. Biol. Chem. 256, 5428 - 5435.

95. Zukin, R.S. and Koshland, D.E. (1976). Science 193, 405 - 408.

96. Plattner, H., Reichel, K. and Matt, H. (1977). Nature 267, 702 - 704.

97. Cooper, P.H. and Stanworth, D.R. (1976). Biochem. J. 156, 691 - 700.

98. Burnstock, G. (1972). Pharmac. Rev. 24, 509 - 581.

99. Trams, E.G. and Lauter, C.J. (1978). Nature 271, 270 - 271.

100. Cockroft, S. and Gomperts, B.D. (1979). Nature 279, 541 - 542.

101. Ronquist, G. and Gunnar, K.A. (1975). Cancer Research 35, 1402 - 1406.

102. Parish, R.W. and Weibel, M. (1980). FEBS Letts. 118, 263 - 266.

103. Landry, Y. and Lehninger, A.L. (1976). Biochem. J. 158, 427 - 438.

104. Coetzee, G.A. and Gevers, W. (1977). Biochem. J. 164, 645 - 652.

105. Malouf, N.N. and Meissnev, G. (1980) J. Histochem. Cytochem. 28, 1286 - 1294.

106. Panagia, V., Lamer, J.M.J., Singal, P.K. and Dhalla, N.S. (1982). Int. J. Biochem. 14, 387 - 397.

107. Dhalla, N.S., Harrow, J.A.C. and Anand, M.B. (1978). Biochem. Pharmacol. 27, 1281 - 1283.

108. Dhalla, N.S., Lee, S.L., Anand, M.B. and Chauhan, M.S. (1977). Biochem. Pharmacol. 26, 2055 - 2060.

109. Harrow, J.A.C., Das, P.K. and Dhalla, N.S. (1978). Biochem. Pharmacol. 27, 2605 - 2609.

110. Ziegelhoffer, A., Anand-Srivastava, M.D., Khandelwal, R.L. and Dhalla, N.S. (1979). Biochem. Biophys. Res. Commun. 89, 1073 - 1081.

111. Dhalla, N.S., Pierce, G.N., Panagia, V., Singal, P.K. and Beamish, R.E. (1982). Basic Res. Cardiol. 77, 117 - 139.

112. McNamara, D.B., Sulakhe, P.V., Singh, J.N. and Dhalla, N.S. (1974). J. Biochem. 75, 795 - 803.

113. Dhalla, N.S., Anand, M.B. and Harrow,J.A.C. (1976). J. Biochem. 79, 1345 - 1350.

114. Takeo, S., Duke, P., Taam, G.M.L., Singal, P.K. and Dhalla, N.S. (1979). Can. J. Physiol. Pharm. 57, 496 - 503.

115. Wikman-Coffelt, J., Zelis, R., Feuner, C. and Mason, D.T. (1973). Biochem. Biophys. Acta. 51, 1097 - 1104.

116. Taussky, H.H. and Shorr, E. (1953). J. Biol. Chem. 202, 675 - 685.

117. Davis, R.J. (1964). Ann. N.Y. Acad. Sci. 121, 404 - 427.

118. Weber, K., Osborn, M. (1969). J. Biol. Chem. 244, 4406 - 4412.

119. Laemmli, U.K. (1970). Nature 227, 680 - 689.

120. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). 193, 265 - 275.

121. Warburg, O. and Christian, W. (1941). Biochem. J. 310, 384 - 421.

122. Butcher, F.R. (1971). Horm. Metab. Res. 3, 336 - 340.

123. Akera, T. (1977). Science 198, 569 - 574.

124. Fleckenstein, A. (1976). Adv. Cardiol. 12, 183 - 191.

125. Klee, C.B., Crouch, T.H. and Richman, P.G. (1980). Ann. Rev. Biochem. 49, 489 - 515.

126. Anand, M.B., Chauhan, M.S. and Dhalla, N.S. (1977). J. Biochem. 82, 1731 - 1739.

127. Morcos, N.C. and Drummond, G.I. (1980). Biochim. Biophys. Acta 598, 27 - 39.

128. Soe, G., Nishi, N., Kakuno, T., Yamashita, J. and Horio, S. (1980). J. Biochem. 190, 473 - 481.

129. Ghanbari, H.A. and McCarl, R.L. (1980). Biochem. J. 191, 627 - 635.

130. Willingham, M.C., Ostlund, R.E. and Pastan, I. (1974). Proc. Nat. Acad. Sci. U.S.A. 4144 - 4148.

131. Heyliger, C.E., Panagia, V. and Dhalla, N.S. (1981). J. Pharmacol. Exp. Therap. 217, 489 - 493.

132. Stewart, P.S., MacLennan, D.H. and Shamoo, A.E. (1976). J. Biol. Chem. 251, 712 - 719.

133. Shamoo, A.E. and Murphy, T.J. (1979). Current Topics in Bioenergetics 9, 147 - 177.

134. Klingenberg, M. (1981) Nature 290, 449 - 453.

135. Jenkins, R.E. and Tanner, M.J.A. (1977). 162, 139 - 147. Biochem. J.

136. Newmark, P. (1980) Nature 284, 659 - 660.

137. Lullman, H. and Peters, T. (1977). Clin. Exp. Pharmacol. Physiol. 4, 49 - 57.