5'-NUCLEOTIDASE OF CARDIAC PLASMA MEMBRANE

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BY

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A thesis submitted to the Faculty of Graduate Studies of
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MASTER OF SCIENCE

TO

My Wife

and

My Parents

with Love

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ABSTRACT

5'-Nucleotidase was examined in sarcolemmal fractions of rat heart which had been isolated by two different methods namely, hypotonic shock-LiBr treatment and sucrose gradient separation. Enzyme activity in the two resulting fractions called HL and S respectively differed significantly in several respects. The fraction HL 5'-nucleotidase had a high affinity for AMP (K_m 35 uM) and ATP was a potent competitive inhibitor. In contrast, the 5'-nucleotidase activity displayed by fraction S showed a low substrate affinity (K_m 130 uM) and sensitivity to ATP. Trypsin and neuraminidase treatments of membranes markedly stimulated 5'-nucleotidase in fraction HL whereas only a modest effect was observed in fraction S. However, under the same experimental conditions both fractions exhibited comparable K^+-pNPPase activities. Considering the possibility that 5'-nucleotidase could be localized at the inside of the vesicular membrane of fraction S, Triton X-100 was added to the assay medium. Even though this treatment resulted in a 60% and 10% increase in enzyme activity in fractions HL and S respectively, there was no apparent change in the K_m of the enzyme in both fractions. Concanavalin A blocked almost completely (95%) 5'-nucleotidase activity in both membrane preparations at a concentration of 2 uM. Hill plots of the data of the concanavalin A inactivation process yielded a coefficient of 2.2 for fraction S which is substantially greater than the value of 1.1 obtained for fraction HL. The vectorial properties of such membrane bound enzyme systems as Na^+-K^+-ATPase, adenylate cyclase and ATP dependent Ca^{2+} binding were used to estimate the relative sidedness of the vesicles present in the fractions. The results suggested that fraction S contained a large proportion of inside-out vesicles with 5'-nucleotidase activity possibly

located at the cytoplasmic side of the membrane. Further, poor lanthanum staining of the vesicles in this preparation suggest the absence of a basement membrane thus a predominance of inside-out vesicles. The activation by trypsin and neuraminidase treatment in fraction HL is discussed in relation to the frequently reported ecto-localization of 5'-nucleotidase. Dense lanthanum staining of vesicles in the HL preparation suggest a predominance of right-side out vesicles. These findings suggest that 5'-nucleotidase can exert its action in vivo at either side of the plasma membrane. Similar evidence has been obtained by Frick and Lowenstein (9) in the perfused rat heart. In both preparations, the enzyme was active over a wide pH range. p-Hydroxymercurybenzoate did not produce any significant effect on 5'-nucleotidase thereby demonstrating that its activity is not dependent on free (-SH) groups.

In another series of experiments it was demonstrated that neither dipyridamole nor papaverine had any apparent effect on the enzyme in fractions HL and S thus suggesting that the mechanisms whereby they increase coronary blood flow do not directly involve 5'-nucleotidase. In contrast caffeine and theophylline partially inhibited the enzyme in both fractions but theophylline appeared to be a more potent inhibitor. Even though the antagonistic effect of caffeine seemed to be the same in both sarcolemmal preparations, the enzyme in fraction S appeared to be more sensitive to theophylline than that present in fraction HL. The inhibition of the enzyme by caffeine and theophylline was of the competitive type in both preparations. These results suggest that by inhibiting 5'-nucleotidase, caffeine and theophylline can influence the production of adenosine in the myocardium.

INTRODUCTION AND STATEMENT OF THE PROBLEM

Although small amounts of 5'-nucleotidase seem to be present in the cytosol (1), histochemical studies have shown that in cardiac tissue this enzyme is localized mainly in the vicinity of the transverse tubular part of the surface membrane (2, 3). Other reports have classified it as an ectoenzyme of the plasma membrane (1, 4 - 8) and it has been suggested that it can act as an adenosine translocase (9). 5'-Nucleotidase, which utilizes 5'-AMP as a substrate, is believed to be responsible for the physiological production of adenosine in cardiac tissue (10). Since the concentration of this metabolite increases in the heart during ischemia, hypoxia, and increased cardiac work (11, 12), adenosine has been proposed to be a link between myocardial energy metabolism and coronary blood flow (10, 11, 13). ATP and ADP inhibit this enzyme, and because of their low Ki, it would appear that 5'-nucleotidase is inhibited in both the hypoxic and adequately oxygenated hearts (14). Hence, it seems that the origin of adenosine in the ischemic or hypoxic heart cannot be explained by considering a change in adenine nucleotide levels.

In addition to its coronary vasodilatory action, adenosine also inhibits the release of norepinephrine in the heart (56), and antagonizes the catecholamine-induced stimulation of cardiac adenylate cyclase (50, 64). Furthermore, there is evidence to show that adrenergic nerve stimulation leads to the formation and release of adenosine in the heart (96). Since this metabolite is utilized in the organ in which it is produced, it can be classified as a local hormone (10). But for adenosine to be effective in this capacity it must be released from the cell. The simplest hypothesis for the biochemical mechanism of release is that adenosine is produced intracellularly and

is then transported across the cell membrane by a carrier-mediated process (facilitated diffusion) according to its concentration gradient. However, the major problem with this hypothesis is the localization of 5'-nucleotidase which has been described as an ectoenzyme. Consequently, adenosine may be produced directly into the extracellular space (10). There is little doubt that in many tissues a considerable proportion of the nucleotidase has access to extracellular 5'-AMP as substrate, but it is not clear if the enzyme has access to only extracellular or to both intra and extracellular AMP (10). Most of the studies on the properties of 5'-nucleotidase in the heart have been studied on the solubilized and purified enzyme (14, 15). But solubilization and purification can influence the behavior of an enzyme relative to its unsolubilized and unpurified form. Hence, the present investigation was undertaken to study the intrinsic properties of 5'-nucleotidase in heart sarcolemmal fractions. Special attention was placed on the possible non-identical orientation of the highly purified membrane vesicles from two different preparations as this can be responsible for the observed difference in the behavior of the enzyme in these preparations.

Recently theophylline has been reported to inhibit the activity of 5'-nucleotidase in the brain and kidney (53, 65) and attenuated the adenosine-induced vasodilation in the brain and heart (45, 47, 49, 63). It was also found to antagonize the inhibition of cardiac adenylate cyclase and myocardial contractile force development by adenosine (64). Caffeine inhibited 5'-nucleotidase in the brain (65) and attenuated the catecholamine-induced stimulation of cardiac adenylate cyclase (50). The mechanisms by which methylxanthines antagonize the effects of

adenosine in the heart are not known. These may partially reside in the inhibition of cyclic nucleotide phosphodiesterase with a subsequent increase of intracellular cAMP levels. Another possible explanation might be a direct influence on 5'-nucleotidase activity as already observed in the brain and kidney. In view of the possibility that such a mechanism could also be operative in the heart, the effects of caffeine and theophylline on cardiac sarcolemmal 5'-nucleotidase were therefore investigated. Furthermore, some experiments were designed to determine whether the elevation of adenosine levels in the extracellular environment by dipyridamole (10, 46) could be partly correlated with changes in 5'-nucleotidase activity. The effect of papaverine on this enzyme was also investigated since this drug is known as an exogenous vasodilator and, like methylxanthines and dipyridamole, is a phosphodiesterase inhibitor (51). The interaction of 5'-nucleotidase with these drugs was investigated by employing heart sarcolemmal preparations obtained by different methods.

REVIEW OF THE LITERATURE

Adenosine exerts important functional and metabolic actions on the myocardium (68) in addition to its well known effect on coronary blood flow (11, 94). The cardiac effects of adenosine include negative chronotropic (69, 94) and inotropic actions (64, 94), increase in glucose uptake by cardiac cells (13, 97) and reduction of myocardial oxygen consumption (70). The first hint that adenosine modifies physiological processes came in 1929 when Drury and Szent-Gyorgi observed that injection of adenosine into mammals lowered the arterial blood pressure, dilated the coronary arterioles, induced sleep and inhibited movements of the small intestine (10, 95). During the following thirty years more attention was given to the pharmacological effects of adenine nucleotides than those of adenosine. In particular, studies were focussed upon ATP, which had not been available in large quantities at the time of Drury's work. Nonetheless, perhaps the most investigated effect of adenosine is on the rate of blood flow. In 1936, Drury suggested that adenosine may be involved in the control of blood flow but this possibility has only been intensively investigated since 1960 (10).

In the course of studying the deamination of nucleotides in heart and skeletal muscle in 1934, Reis discovered that adenosine is produced by the hydrolysis of 5'-AMP (71) and deduced that the reaction was catalysed by an enzyme specific for 5'-nucleotidases now known as 5'-nucleotidase (10). This enzyme catalyses the dephosphorylation of 5'-AMP according to the reaction (31):

$$\text{5'-AMP} \xrightarrow{\text{5'-nucleotidase}} \text{adenosine and orthophosphate}$$

Subsequent to his deduction of the presence of 5'-nucleotidase in

animal tissue, Reis then undertook the task of characterizing this enzyme. His experiments however, were performed with impure preparations of the enzyme thus it is difficult to draw any meaningful conclusions from such studies (71). Since then, the advent of better techniques for studying enzymes have greatly improved the accuracy in the characterization of 5'-nucleotidase.

Adenosine is produced, released, taken up and metabolized by most animal tissues. A number of pathways could be involved in the metabolism of this nucleoside; however, some of these are of little or no quantitive significance (10). Acid or alkaline phosphatases could be involved in the formation of adenosine since they catalyse the hydrolysis of 5'-AMP to adenosine. However, these enzymes show no specificity for 5'-AMP and their activities at neutral pH are too low to be of quantitive physiological importance in adenosine production (10, 73). Similarly, purine nucleotide phosphorylase which could produce adenosine from adenine has a very low activity (10, 74). On the other hand, 5'-nucleotidase is specific for 5'-nucleotides (10, 71); its pH optimum is close to the physiological pH and its maximal activity can more than adequately account for known rates of adenosine formation (10, 76).

A. Cellular Localization and Characteristics of 5'-Nucleotidase:

Perhaps the earliest attempt to determine the intracellular localization of 5'-nucleotidase was that of Novikoff and his associates in 1953 (77). By using an elaborate scheme of centrifugation and separation of rat liver suspensions into different subcellular fractions, these workers reported that most of the enzyme was associated with their nuclear fraction. The distribution of 5'-nucleotidase was estimated from charts and expressed as a fraction of the activity in the homogenate

(71). Electron microscopic studies however, have demonstrated the presence of 5'-nucleotidase in the plasma membranes of practically all mammalian cells (71, 78). Because of this finding this enzyme is now widely used as an enzymatic marker during purification of these membranes (78). 5'-Nucleotidase is a glycoprotein (61, 79, 80). In the mouse liver, it has a molecular weight of 140-150,000 daltons, and is composed of two probably identical subunits (61, 79), but the molecular weight and structure of the cardiac enzyme are unknown (61).

There are marked species differences with respect to the cardiac 5'-nucleotidase activity. Experiments performed by Baer et al (35) have demonstrated that rat hearts contain 100 times the activity found in rabbit heart. Their experiments also revealed that the activity of 5'-nucleotidase in the hearts of dog, sheep and guinea pig is considerably lower than the activity found in rat heart. Further, Nakatsu and Drummond (15) have reported that turtle and pigeon ventricles contain no detectable 5'-nucleotidase activity. These findings clearly demonstrate that the activity of this enzyme is neither related to the size of the animal nor to animals that experience large and rapid changes in heart work load, for example birds as compared to animals like reptiles that maintain constant loads on their hearts (15).

Rat ventricular 5'-nucleotidase possesses a broad substrate specificity for nucleoside 5'-monophosphates. However, 5'-AMP is the preferred substrate (14). According to Nakatsu and Drummond (15), phosphate esters not bearing base moieties were not attacked by this enzyme. Thus, ribose-5-phosphate, glucose-6-phosphate, fructose-1-phosphate, ribulose-5-phosphate and galactose-6-phosphate do not appear to serve as substrates. Phosphate is not a substrate; neither is p-nitrophenylphosphate (15) which serves as a substrate for non-specific

phosphatases.

5'-Nucleotidase activity in the heart appears to be influenced by divalent cations. As indicated by Nakatsu and Drummond (15), the activity of this enzyme was greatly increased in the presence of Mg^{2+}, Ni^{2+} and Mn^{2-} at concentrations of 16, 2 and 1 mM respectively. Of these elements, Mn^{2+} appears to be the more potent stimulator; however, it was inhibitory at concentrations above 2 mM. Ca^{2+} had only slight stimulatory effect but does not appear to affect the activity of the enzyme in the presence of Mg^{2+}. Sullivan and Alpers (14) have confirmed the stimulatory effect of Mg^{2+} and Ca^{2+} on 5'-nucleotidase activity. In contrast to these findings are the earlier observations of Edwards and Maguire (81) who reported that both Mg^{2+} and Ca^{2+} inhibited the activity of 5'-nucleotidase in the heart. The general agreement is however, that Mg^{2+} is required for the maximal activity of cardiac 5'-nucleotidase.

The inhibition of 5'-nucleotidase by ATP and ADP has been well documented by several groups of investigators. The type of inhibition exerted by these nucleoside di-and-triphosphates is uncertain. ATP inhibition has been reported to be competitive (35), mixed (61), or noncompetitive (61, 80), while ADP inhibition is said to be competitive (14, 80, 81) or noncompetitive (15). According to Olsson and Patterson (61), this conflict is probably due to differences in experimental conditions, particularly pH and Mg^{2+} concentration. Other known inhibitors of 5'-nucleotidase activity are methylxanthines (53, 65), orthophosphate (one of the products of the dephosphorylation of 5'-AMP by this enzyme (15)) and the synthetic compound γ, β-methylene adenosine diphosphate (AOPCP). The type of inhibition exerted by AOPCP, an ADP analog, is strongly dependent on pH, being of the mixed type below pH 7 and competitive at pH 7 and above (61).

B. Regulation of 5'-Nucleotidase in vivo:

One approach to understanding the regulation of adenosine production is to define how 5'-nucleotidase is regulated in vivo. Cardiac tissue appears to contain much more 5'-nucleotidase than is required to account for the observed rates of adenosine production in beating hearts. Dog heart homogenates for example contain enough of this enzyme to produce about 130 nmoles adenosine/g per min (61), yet oxygenated hearts produce less than 1 nmole/g per min (61, 80) and this figure increases only about 6-fold during coronary occlusion (61, 90). This disparity between the actual and potential rates of adenosine production suggest that 5'-nucleotidase is markedly inhibited in vivo (61). Several factors that can contribute to this relatively low apparent activity have been suggested by different investigators.

When Baer, Drummond, and Duncan (35) found that ATP inhibited 5'-nucleotidase, they proposed that this inhibition accounted for the in vivo control of the enzyme. According to this postulate, hypoxia should lead to an increase in the production of adenosine because it causes ATP levels to fall, thereby relieving the inhibition of the enzyme. Further, the conversion of ATP to AMP in hypoxic heart would provide more substrate for the enzyme. However, current evidence does not appear to support this hypothesis (61). ATP levels in heart do not change for at least 15 seconds after coronary artery ligation (61, 91), whereas adenosine levels increase after as little as 5 seconds of coronary artery occlusion (61, 90). Further, a decrease in ATP necessarily implies an increase in ADP, which has been observed within 15 seconds after coronary occlusion (61, 92). ADP inhibits 5'-nucleotidase even more strongly than ATP (14, 80) which should increase rather than decrease the inhibition of the enzyme (61).

According to Olsson and Patterson (61), the ionic composition of the microenvironment of the enzyme may also influence in vivo activity of 5'-nucleotidase. Because the enzyme is located in a membrane through which there are very important ion flexes which ultimately determine cardiac performance, it may be very susceptible to this sort of local chemical control. Sullivan and Alpers (14) have proposed that the regulation of 5'-nucleotidase in vivo may depend on the deinhibition by magnesium. In this regard it was demonstrated that the inhibition imposed by either ATP or ADP was not relieved by a number of metabolic intermediates known to accumulate in the hypoxic heart. However, magnesium, one of the many divalent cations required for the activity of this enzyme relieved the nucleotide-induced inhibition completely. This relief was in part caused by the formation of nucleotide-magnesium complex which was less inhibitory. Further increases in magnesium relieved the inhibition imposed by the complexes (14).

As indicated by Arch and Newsholme (76) one mechanism for lowering the activity of 5'-nucleotidase in vivo maybe that the concentration of substrate (5'-AMP) is well below the K_m value of the enzyme. Thus, the concentration of AMP in the tissue is similar to or below the K_m value of the nucleotidase. Moreover, much of this AMP may be bound to proteins and/or localized within the mitochondria (76, 93). Such conditions could play an important role not only in decreasing the activity of 5'-nucleotidase in vivo but also in modifying the activity of the enzyme under certain physiological conditions in order to produce a change in the steady-state concentration of adenosine (76).

C. Physiological Role of 5'-Nucleotidase:

A physiological role has not been firmly established for

5'-nucleotidase although a function in adenosine production has been proposed. Physiological concentrations of adenosine cause vasodilation in a number of mammalian organs including the heart, skeletal muscle, brain, intestine and adipose tissue (82, 83, 84). In contrast, adenosine produces vasoconstriction in the kidney (53) and liver (10). Further, adenosine inhibits lipolysis in adipose tissue (85) and has been implicated as neurohumoral agent in the brain (5). Adenosine also influences myocardial contractility in several mammalian species (10) and low concentrations of this nucleoside inhibit platelet aggregation (10, 86).

It has been shown by several investigators that the concentration of 5'-AMP influences the activity of at least three important enzymes in the glycolytic pathway and citric acid (71, 87, 88). This has been termed the adenylate control hypothesis, and some of the most important enzymes this control are glycogen phosphorylase, phosphofructokinase and fructose-1-6-diphosphatase. It is conceivable that the intracellular activity of 5'-nucleotidase may influence the concentration of 5'-AMP, and thus the activity of these enzymes (71).

There have been several observations that various hormones alter 5'-nucleotidase activity in various tissues, particularly those tissues that are target organs. For example, Gepts and Toussaint reported that the histochemical staining reaction for 5'-nucleotidase was intensified in the pancreatic islets of cortisone-treated rats (71). In 1958, Reid and Stevens (89) demonstrated that hypophysectomy in rats resulted in a decrease in liver nucleotidase activity. Although the hormonal control of the myocardial 5'-nucleotidase is poorly understood, it must be emphasized that further investigations about the behavior of 5'-nucleotidase in myocardium is greatly warranted mainly because it

plays a significant role in adenosine production which influences myocardial contractility. The information thus gathered can be of some importance in assigning a specific physiological role for this seemingly important enzyme in the heart.

METHODS

A. Animals

Male Sprague-Dawley rats (300-400 grams) were used for all experiments in this study. After decapitation, the hearts were quickly excised and washed free of blood with ice-cold saline solution.

B. Preparation of Plasma Membranes

i) Hypotonic shock-LiBr treatment method

Rat heart ventricles were washed thoroughly, diced with a pair of scissors, and homogenized in 10 volumes of 10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA in a Waring Blender for 1 min. (30 sec. x 2), with an interval of 1 min. The homogenate was filtered through gauze and centrifuged at 1,000xg for 10 min. The sediment was suspended in 25 volumes of the above Tris-buffer and stirred in a cold room for 15 min. and centrifuged at 1,000xg for 10 min. The residue was then resuspended in 25 volumes of 10 mM Tris-HCl, pH 8.0, stirred for 15 minutes and centrifuged at 1,000xg for 10 min. The sediment was suspended in 10 mM Tris-HCl, pH 7.4, stirred for 15 min. and centrifuged at 1,000xg for 10 min.; this step was repeated again. The sediment was suspended in 25 volumes of 10 mM Tris-HCl, pH 7.4, extracted with 0.4 M Libr for 30 min. and centrifuged at 1,000xg for 10 min. The sediment was resuspended in 25 volumes of 10 mM Tris-HCl, pH 7.4, stirred for 10 min., then centrifuged for 10 min. The residue was suspended in 0.6 M KCl, 10 mM Tris-HCl, pH 7.4 and stirred for 15 minutes and centrifuged at 1,000xg for 10 min. The sediment was again washed with 10 mM Tris-HCl, pH 7.4, stirred for 10 min. and centrifuged for 10 mins. This sediment was suspended in 1 mM Tris-HCl, pH 7.4 and used. All the

above steps were performed at 0^{o} - 4^{o} C. For convenience of discussion these membrane vesicles will be called fraction HL. This isolation procedure was carried out essentially according to the method of McNamara et al (16) except that stirring times were reduced by 15 min. and an additional 0.6 M KCl 10 mM Tris-HCl pH 7.4 extraction step was performed before the last washing with 10 mM Tris-HCl, pH 7.4 (17).

ii) Discontinuous sucrose density gradient centrifugation

Rat heart ventricles were washed thoroughly; diced with a pair of scissors and homogenized in 0.25M sucrose by a 15 sec. burst at a setting 9 of polytron PT 10. The homogenate was layered on top of a gradient 39%, 49% and 80% (W/W) sucrose prepared in 50 ml tubes. These were then centrifuged in an SB-116 rotor for 90 min. at 55,000xg. The plasma membranes appeared mainly at the 0.25M and 39% interphase. Microsomes were located in the vicinity of interphase 39%/49%, and the dense mitochondrial fraction in the area of the 45%/80% interphase. Each fraction was collected with a Pasteur pipette, diluted with 0.25M sucrose then centrifuged for 20 min. at 90,000xg in an A-211 rotor. The final suspension of each particular set of pellets was in a small volume of 0.25M sucrose. All of the above steps were performed at 0^{o} - 4^{o} C. For convenience, this sarcolemmal preparation will be referred to as fraction S. This isolation procedure was carried out in principle according to the method previously described by Kidwai et al (18, 24). The modifications included the use of a discontinuous gradient, and the omission of the 100,000xg centrifugation of the homogenate. Both fractions (HL and S) could be stored at -80^{o}C for about 3 weeks without loss of 5'-nucleotidase activity.

C. Isolation of Mitochondria

Thoroughly washed rat heart ventricles were minced with a pair of scissors. The tissue was homogenized in 10 volumes of 0.18 M KCl-10mM EDTA-0.5% albumin. pH 7.4 in a Waring Blender for 2 x 10 sec with a 1 min. interval. The homogenate was filtered through 4 layers of gauze and centrifuged at 1,000xg for 10 min. The pellets were discarded and the supernatant was centrifuged at 10,000xg for 20 min. The pellets were washed in 10 volumes of the above mentioned KCl-buffer and centrifuged at 1,000xg for 10 min. The residue was again discarded and the supernatant further centrifuged at 8,000xg for 10 min. The pellets were thoroughly washed and suspended in a small volume of 50 mM KCl-20mM Tris-HCl, pH 6.8 and used. This isolation procedure was carried out according to the method of Sordahl et al (39) at 0^{o} - 4^{o}C. A teflon-glass homogenizer was used for homogenization of pellets.

D. Isolation of Microsomes

After the rat heart ventricles were thoroughly washed, they were minced with a pair of scissors. The tissue was homogenized in 10 volumes of 10 mM $NaHCO_3$-5mM NaN_3-15mM Tris-HCl, pH 6.8 in a Waring Blender for 45 sec. The homogenate was filtered through 4 layers of gauze then centrifuged at 10,000xg for 20 min. The residue was discarded and the supernatant further centrifuged at 40,000xg for 45 min. The pellets were suspended in 10 volumes of 0.6 M KCl-20mM Tris-HCl, pH 6.8 and centrifuged at 40,000xg for 45 min. Finally, the pellets were suspended in 0.25 sucrose-20mM Tris, pH 6.8 and used. This method is essentially the same as that described by Sulakhe and Dhalla (40). Isolation procedures were performed at 0^{o} - 4^{o} C.

E. Differential Centrifugation

Rat heart ventricles, after washing thoroughly to remove blood, were minced then homogenized with 10 volumes of 0.25M sucrose, 10mM Tris-HCl, pH 7.4 in a Waring Blender for 2 x 10 sec. The homogenate, after filtration through 4 layers of gauze, was centrifuged at 1,000xg for 10 min. The residue was washed, suspended in the homogenizing buffer and centrifuged at 1,000xg for 10 min. This step was repeated twice and the washed residue was suspended in the same buffer to obtain the sarcolemmal fraction. The combined supernatants were then centrifuged at 10,000xg for 20 min to obtain the mitochondrial fraction which was washed once in the sucrose buffer, suspended and centrifuged at 1,000xg for 10 min. The residue was discarded and the supernatant further centrifuged at 8,000xg for 15 min to obtain the mitochondrial sediment which was then resuspended in the sucrose buffer. The post mitochondrial supernatant was then further centrifuged at 40,000xg for 1 hour to obtain heavy microsomal fraction. This was suspended in 0.6M KCl-10mM Tris-HCl, pH 7.0, centrifuged at 40,000xg for 45 min and suspended in the sucrose buffer. This was the heavy microsomal fraction. The post 40,000xg supernatant was further centrifuged at 100,000xg for 1 hour to obtain light microsomes. Final suspension of this fraction was in the sucrose buffer. The post 100,000xg supernatant and the above fractions were used for studying the activity of 5'-nucleotidase. This procedure for the isolation of crude subcellular fractions is essentially the same as that outlined by Sulakhe and Dhalla (41).

F_1 Treatment of Membranes with Trypsin and Neuraminidase

Aliquots of membrane protein were incubated with either enzyme or with a buffer containing 50 mM Tris pH 7.5 and 20 mM KCl pH 7.5 at

37°C. After 20 minutes, 50 ul of treated membranes were transferred to test tubes containing the assay mixture that was pre-incubated for 3 minutes at 37°C. These tubes were then used to determine the activity of 5'-nucleotidase. In the case of trypsin treatment 0.6 mg trypsin inhibitor was added before the transfer. Control preparations were treated identically without addition of any trypsin or neuraminidase.

F_2 Alternative Procedure for Trypsin Treatment

Small amounts of membrane proteins were incubated with this proteolytic enzyme for 20 minutes at 37°C. The reaction was terminated with trypsin inhibitor. A small aliquot was then transferred for the direct assay of 5'-nucleotidase. The remainder of the incubation mixture was centrifuged at 48,000xg for 30 minutes at 0° - 4°C. The supernatant was collected and the pellets washed in buffer (50 mM Tris pH 7.5 and 20 mM KCl). Final suspension of the pellets was in the same medium. Supernatant, pellet suspension and uncentrifuged sample were assayed for 5'-nucleotidase.

G. Enzymatic Determinations

i) 5'-Nucleotidase

This enzyme was assayed essentially according to the method of Avruch and Wallace (19). The assay was performed at 37°C in a 200 ul volume containing 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0, 200 uM (U-^{14}C)-AMP as substrate and 2 mM dicyclohexylammonium p-nitrophenylphosphate as substrate for non-specific phosphatases. After a pre-incubation period of 4 min, the reaction was initiated by adding 50 ul of membrane suspension. It was terminated 10 min later with 50 ul of 0.25M $ZnSO_4$. Subsequently, 50 ul of Ba $(OH)_2$ was added for the precipitation of AMP.

After centrifugation, 200 ul of the supernatant containing adenosine was added to 10 ml of scintillant and radioactivity determined in a liquid-scintillation counter.

ii) Adenylate Cyclase

The adenylate cyclase activity was assayed at 37° C in a total volume of 0.15 ml containing 50 mM Tris-HCl, pH 7.5, 8 mM caffeine, 5 mM KCl, 20 mM creatine phosphate, 15 mM $MgCl_2$, 0.53 mg/ml creatine kinase and 0.4 mM ATP-^{14}C. The reaction was started by the addition of membrane protein and stopped by boiling the tubes for 3 min under conditions preventing evaporation, with prior addition of cold cyclic AMP (final concentration 1.5 - 2.0 mM). The tubes were centrifuged and 100 ul of the clear supernatant spotted on Whatman No. 3MM paper for descending chromatography, using 1M ammonium acetate: 95% ethanol (15:35). After drying, the cyclic AMP spot was visualized by ultra-violet light. The area containing cyclic AMP was cut and counted in 20 ml of scintillant in a liquid scintillation counter. The above method is essentially similar to that described by Drummond and Duncan (20) except that the ATP regenerating system pyruvate kinase-phosphoenol pyruvate was replaced with creatine phosphate-creatine kinase system.

iii) Na^+-K^+-ATPase

For the Na^+-K^+-ATPase activity, the membrane fraction was incubated in a total volume of 1 ml containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 4 mM $MgCl_2$, 100 mM NaCl and 10 mM KCl in the presence or absence of 2 mM ouabain. After 4 min of pre-incubation at 37° C, the reaction was

started by the addition of 40 mM Tris-ATP. The incubation time was 10 min then the reaction was stopped by the addition of 1 ml of 12% cold trichloroacetic acid (TCA). After centrifugation, Pi in the clear supernatants was determined according to the method of Taussky and Shorr (21). The difference between the total (Na^+-K^+-ATPase and Mg^{2+}-ATPase) and the Mg^{2+}-ATPase activities was taken to be due to Na^+-K^+-ATPase activity. Ouabain-sensitive Na^+-K^+-ATPase was estimated as the difference between the total ATPase activity in the presence and absence of 2 mM ouabain.

iv) Mg^{2+}-ATPase

The assay for the Mg^{2+}-ATPase activity was performed in a total volume of 1 ml containing 50 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 4 mM Tris-ATP and 1 mM EDTA. After 4 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% TCA. After centrifugation, Pi in the clear supernatants was determined according to the method of Taussky and Shorr (21). The ATP hydrolysis that occurred in the absence of Mg^{2+} was subtracted in order to calculate the activity due to Mg^{2+} stimulated ATPase.

v) K^+-pNPPase

For the estimation of K^+-pNPPase activity, the membrane protein was incubated in total volume of 1 ml containing 50 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 1 mM EDTA, 200 mM KCl, 2 mM ouabain and 3 mM p-nitrophenylphosphate. After 4 min of pre-incubation at 37° C, the reaction was started by the addition of the p-nitrophenylphosphate;

the incubation was stopped after 30 min by adding 0.2 ml of 24% TCA. After centrifugation, the supernatant containing p-nitrophenol was mixed with 2 ml of 1M Tris, pH 10.5, and thereafter samples were measured at 400 mu (E=15.1 $umol^{-1}cm^{-1}$). The difference between the activity of the enzyme in the presence and absence of K^+ is referred to as K^+-stimulated phosphatase. Ouabain sensitivity of this enzyme was estimated as the difference between its activity in the presence of 2 mM ouabain coupled with the presence and absence of K^+. This assay procedure is essentially the same as that described by Lamers et al (22).

vi) Calcium Binding

The ATP-dependent calcium binding by the sarcolemmal fractions was measured in a medium consisting of 100 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-maleate, pH 6.8, 0.1 mM $^{45}Ca\ Cl_2$, 2 mM ATP in a total volume of 1 ml. The fractions were pre-incubated for 4 min at 25° C in the presence of ATP. The reaction was started by the addition of $^{45}CaCl_2$ and stopped 5 min later by millipore filtration. The amount of ^{45}Ca in 0.1 ml of filtrate was analyzed in 10 ml of scintillant in a liquid scintillation counter.

H. Electron Microscopy

Pellets of treated and untreated membranes were fixed in 2% glutaraldehyde in 0.1M phosphate buffer for 1 hour at 4° C, pH 7.3. The material was washed in the same buffer for 4 hours and post fixed in 1% osmium tetraxide (OSO_4) for 1 hour. Dehydration was done in ethanol series followed by embedding in Epon. Thin sections were

double stained with lanthanum and examined in the electron microscope Carl Zeiss EM9.

I. Protein Content

Protein concentration was determined by the Lowry method (42) with bovine serum albumin as standard.

J. Chemicals

(U-^{14}C)-AMP (1.25 m Ci/mg) and (U-^{14}C)-ATP (0.08 m Ci/mg) were supplied by New England Nuclear Corporation (Canada). Disodium-phosphocreatine, creatine phosphate, 3'-5'-cyclic AMP, 5'-AMP dicyclohexylammonium p-nitrophenylphosphate, ATP, neuraminidase, concanavalin A, trypsin inhibitor, papaverine and theophylline were purchased from Sigma Chemical Corporation (St. Louis, Mo.). Other chemicals were of analytical grade.

RESULTS

1. Characterization of Heart Sarcolemma 5'-Nucleotidase

A. Subcellular Distribution of 5'-Nucleotidase in Rat Ventricular Myocardium

The subcellular distribution of 5'-nucleotidase activity in the heart homogenate was studied by separating different fractions by the differential centrifugation procedure and the results are shown in Table 1. The 40,000xg fraction, which contained fragments of the sarcoplasmic reticulum (crude microsomal fraction), was found to exhibit the highest specific activity but the total enzyme activity in this was about 8%. A large amount of the total enzyme activity (about 65%) was present in the 1,000xg fraction which is believed to contain sarcolemmal fragments, nuclei, myofibrils, intact cells and connective tissue. These contaminants might be responsible for some of the observed 5'-nucleotidase activity in this crude sarcolemmal fraction especially since the total activity of the enzyme in each of the purified sarcolemmal fraction (Table 2) was lower than that observed in the 1,000xg fraction. The particulate nature of the 5'-nucleotidase in heart is evident since the enzyme activity in the 100,000xg fraction, the soluble fraction was almost negligible. In another series of experiments, sarcolemmal, mitochondrial and microsomal fractions were prepared by two different methods and the results concerning their specific as well as relative enzyme activities are given in Table 2. Although the specific activity in the purified microsomal fraction was 1.5 to 2 fold of that in the purified sarcolemmal fraction, the protein yield of the sarcolemma was about 3 fold of that for the microsomal fraction. In view of the

TABLE I

Distribution of 5'-nucleotidase in fractions obtained by differential centrifugation of rat heart homogenate which was obtained from 4 grams of tissue. Values given are the means ± S.E.M. of 4 experiments.

FRACTIONS	5'-Nucleotidase			Protein Yield (mg)
	Specific Activity nmol/min/mg	Total Activity nmol/min.	% Activity	
Homogenate	23.1 ± 0.8	9369.8 ± 1.2	100	405.5 ± 1.2
1,000xg	26.6 ± 0.8	6091.4 ± 1.6	65.0 ± 2.1	229.0 ± 0.5
10,000xg	29.0 ± 1.5	861.3 ± 2.4	9.2 ± 3.4	29.7 ± 1.2
40,000xg	76.5 ± 0.2	810.9 ± 0.9	8.7 ± 1.9	10.6 ± 0.4
100,000xg	54.1 ± 2.2	227.2 ± 1.8	2.3 ± 1.4	4.2 ± 1.1
100,000xg Supernatant	3.5 ± 0.1	4.9 ± 0.2	0.1 ± 0.01	1.4 ± 0.1

TABLE 2

Specific activity of 5'-nucleotidase in different subcellular fractions obtained by different methods. The specific activity in the homogenate is reported in Table I. Values given are the means ± S.E.M. of 4 experiments.

FRACTIONS AND METHODS	5'-Nucleotidase		Protein Yield	
	Specific Activity nmol/min/mg	Relative Specific Activity	Protein Yield (%)	mg
A. Discontinuous Sucrose Density Gradient				
i. Sarcolemma (Fraction S)	206 ± 14.0	8.9 ± 0.5	1.6 ± 0.1	6.5 ± 0.1
ii. Mitochondria	32.2 ± 2.2	1.4 ± 0.3	7.1 ± 0.8	28.8 ± 0.8
iii. Microsomes	324.1 ± 4.1	14.0 ± 0.5	0.5 ± 0.3	2.0 ± 0.3
B. Hypotonic Shock-LiBr Sarcolemma (Fraction HL)	64.0 ± 3.0	2.8 ± 0.1	3.7 ± 0.3	15.0 ± 0.3
C. Mitochondria (Sordahl et al)	17.0 ± 2.5	0.7 ± 0.1	5.3 ± 0.5	21.5 ± 0.5
D. Microsomes (Sulakhe and Dhalla)	113.0 ± 2.2	5.0 ± 0.1	1.2 ± 0.3	4.7 ± 0.3

presence of sarcolemmal enzymes such as 5'-nucleotidase in other subcellular fractions such as mitochondria and microsomes (43), it was decided to further characterize the enzyme present in the sarcolemmal fractions obtained by two different methods. In this regard, it is pointed out that these purified sarcolemmal preparations contained minimal activities of marker enzymes for mitochondria, liposomes and microsomal fractions (16, 23). In contrast, Na^+-K^+-ATPase, adenylate cyclase and 5'-nucleotidase were enriched several folds relative to the homogenate in both fractions (Tables 2 and 3). It is noteworthy that the activities of 5'-nucleotidase, Na^+-K^+-ATPase and adenylate cyclase were higher in the sarcolemmal preparations (fraction S) obtained by the sucrose-density gradient method in comparison to those in the membranes (fraction HL) obtained by the hypotonic shock-LiBr treatment method.

B. Kinetic Properties of 5'-Nucleotidase

The enzyme activities of both HL and S fractions were studied by employing different concentrations of 5'-AMP. Figures 1 and 2 show that the K_m values for the substrate 5'-AMP were significantly different in both fractions. The K_m (35 uM) observed in fraction HL was very close to those reported for the partially purified (23 uM) and solubilized enzyme (21 uM) from cardiac tissue (14, 15). In contrast, the K_m (130 uM) obtained in fraction S differed considerably. ATP appeared to be a competitive inhibitor of the enzyme in the two preparations as indicated by the Lineweaver-Burke plots constructed in the concentration range of 40-200 uM AMP in the presence and absence of 200 uM ATP. There was a significant change in K_m for the enzyme in both sarcolemmal preparations in the presence of

TABLE 3

Specific activities of marker enzymes in homogenate and sarcolemmal fractions obtained from rat ventricles. Values given are the means ± S.E.M. of at least 4 experiments.

FRACTION	Na^+-K^+-ATPase (umol/h/mg)	Adenylate Cyclase (pmol/min/mg)	Protein Yield (%)
Homogenate	0.6 ± 0.3	40.2 ± 0.6	100
Membrane Fraction HL	18.3 ± 2.0	343 ± 25	3.7 ± 0.3
Membrane Fraction S	25.0 ± 6.3	502 ± 12	1.1 ± 0.1

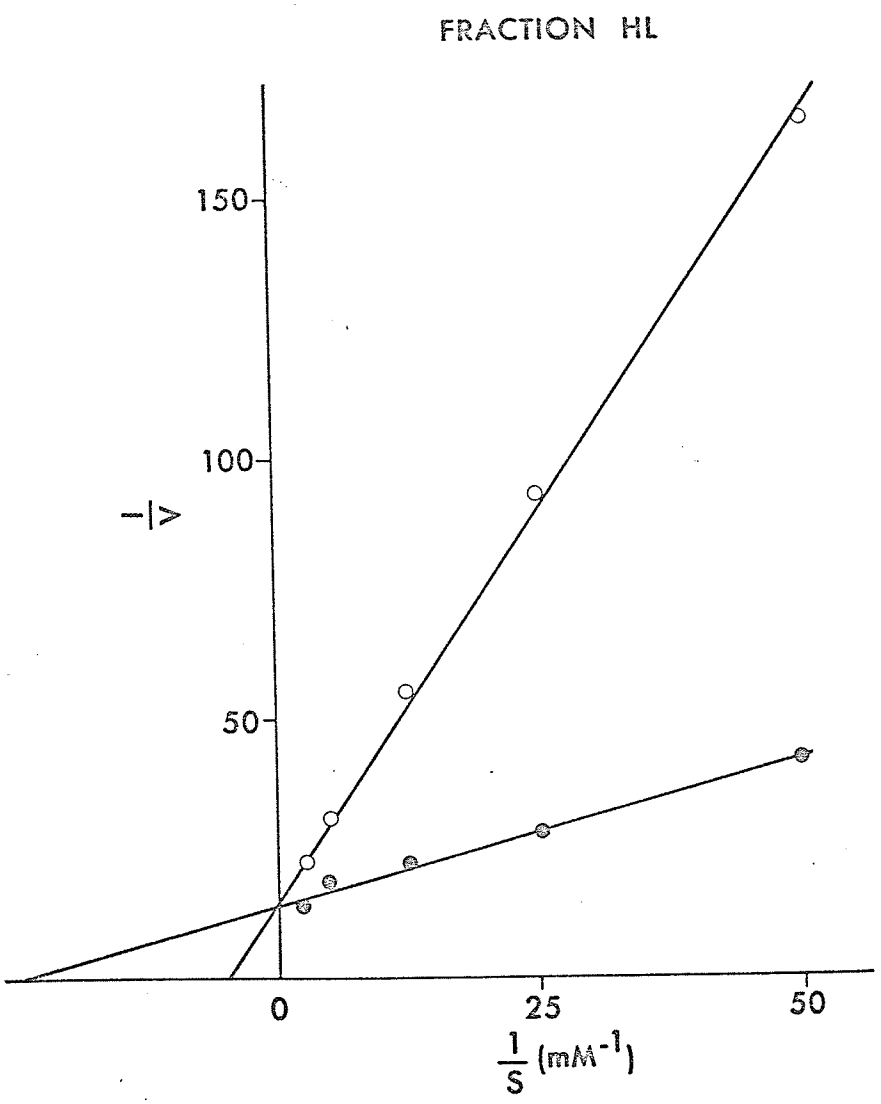

FIGURE 1 Inhibition of 5'-nucleotidase by ATP in fraction HL. The enzyme was assayed by incubating portions with 20-400 uM AMP. The reactions were carried out in the absence (●—●) and presence (○—○) of 200 uM ATP. Lineweaver-Burke plots were constructed from the data. V, nmol of adenosine formation. min^{-1}. mg^{-1}. S, AMP. Results are the means of 2 experiments. The assay procedure was the same as in Methods.

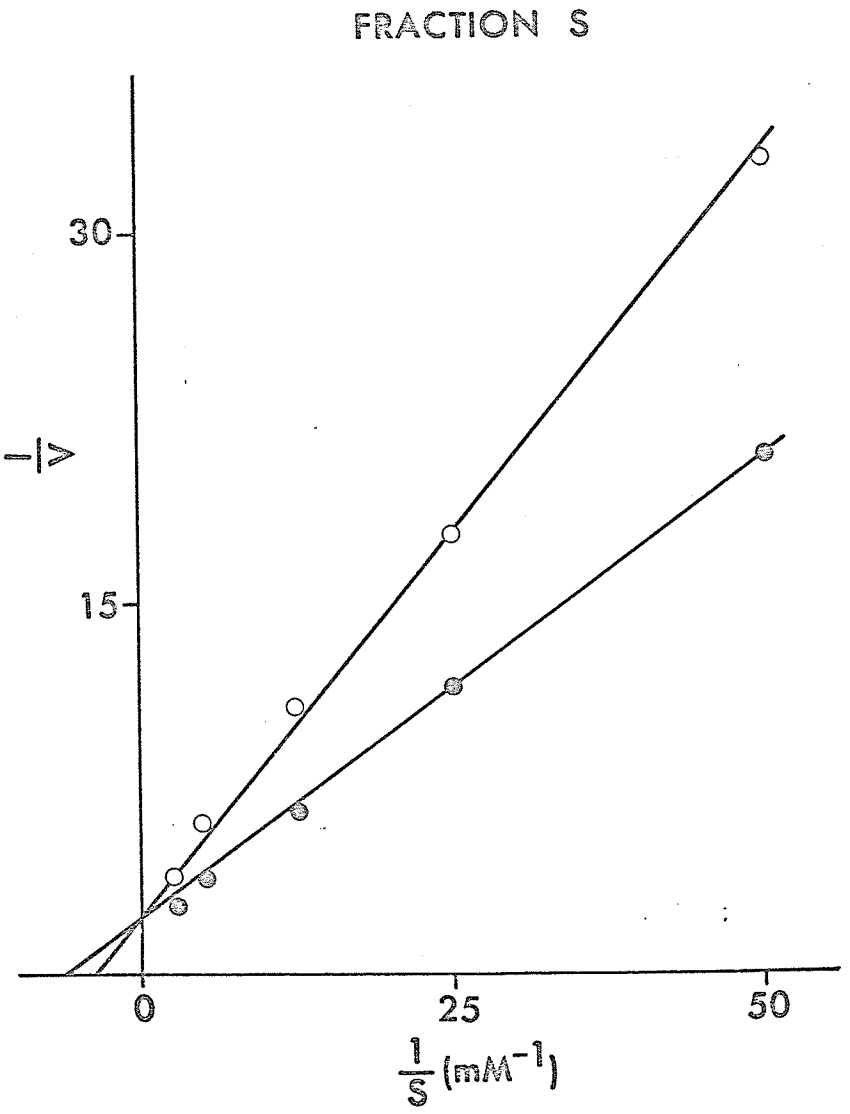

FIGURE 2 Inhibition of 5'-nucleotidase by ATP in fraction S; (●—●) control and (○—○) in the presence of 200 uM ATP. The enzyme was assayed as described in Methods and Table 1. V, nmol adenosine formation. min^{-1}. mg^{-1}. S, AMP. Results are the means of 2 experiments.

ATP; in fraction HL it changed from 35 uM to 200 uM whereas in fraction S the change was from 130 uM to 250 uM. One possible explanation for this observed difference is that 5'-nucleotidase in fraction S might be localized at the inside of intact vesicles that have retained the relatively low permeability to adenine nucleotides and this may hinder the access of AMP. Considering the possibility that the two fractions might not have the same overall sidedness, the effect of treating the pellets with trypsin and neuraminidase was subsequently studied. Both sarcolemmal fractions employed here have been reported to be comprised of membrane vesicles (16, 18, 23).

C. Treatment of Membranes with Trypsin and Neuraminidase

The treatment with trypsin or neuraminidase showed a clear-cut difference of enzymatic activity in the two fractions (Table 4). Whereas there was a strong increase of specific activities in fraction HL, 5'-nucleotidase in fraction S appeared to be relatively insensitive to these treatments. Of interest too was the finding that although the trypsin treatment completely inactivated K^+-pNPPase, an enzyme used to measure the dephosphorylation rate of the membrane bound phosphorylated Na^+-K^+-ATPase (22), in both fractions, the neuraminidase treatment had no effect on this enzyme (Table 4). Thus, these results show that the behavior of 5'-nucleotidase upon trypsin and neuraminidase treatments differed from that of other membrane bound enzymes studied under the same conditions and as such, can suggest that the active site of 5'-nucleotidase in the membrane is different in the two fractions. Moreover, for Na^+-K^+-ATPase it is now generally accepted that splitting of ATP occurs due to the concerted ion activation at both sides of the membranes and accordingly it can be expected that such an enzyme might

TABLE 4

EFFECT OF PRETREATMENT OF MEMBRANES WITH TRYPSIN OR NEURAMINIDASE ON THE ACTIVITY OF 5'-NUCLEOTIDASE AND K^+-pNPPASE.

Membrane protein (0.2 mg) was incubated with 0.2 mg trypsin or neuraminidase. Trypsin reaction was inhibited with 0.6 mg trypsin inhibitor. Because of the sensitivity of the assay, the pretreatment for K^+-pNPPase assay was done with 5 fold the amount of membrane protein as well as trypsin and neuraminidase. Treatment of membranes was the same as in Methods.

TREATMENT		Fraction HL		Fraction S	
		5'-Nucleotidase	K^+-pNPPase	5'-Nucleotidase	K^+-pNPPase
		% of control		% of control	
Trypsin	A.	275.2	1.1	110.7	2.2
	B.	272.8	0.8	107.3	1.7
Neuraminidase	A.	204.6	89.6	108.9	118.0
	B.	201.3	84.2	112.9	120.0

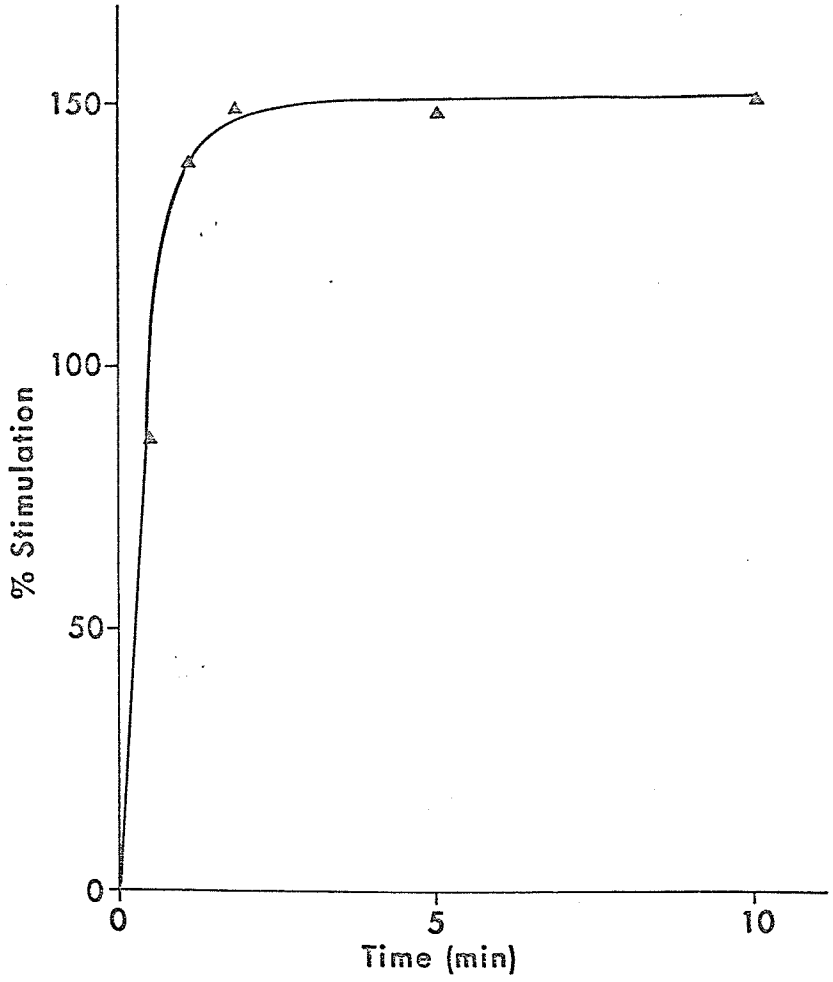

FIGURE 3 Time course study of the effect of trypsin on 5'-nucleotidase in fraction HL. The assay system was carried out with 50 ug membrane fraction, 200 ug trypsin/mg protein and 250 ug trypsin inhibitor as described in Methods. Each value is an average of 3 experiments.

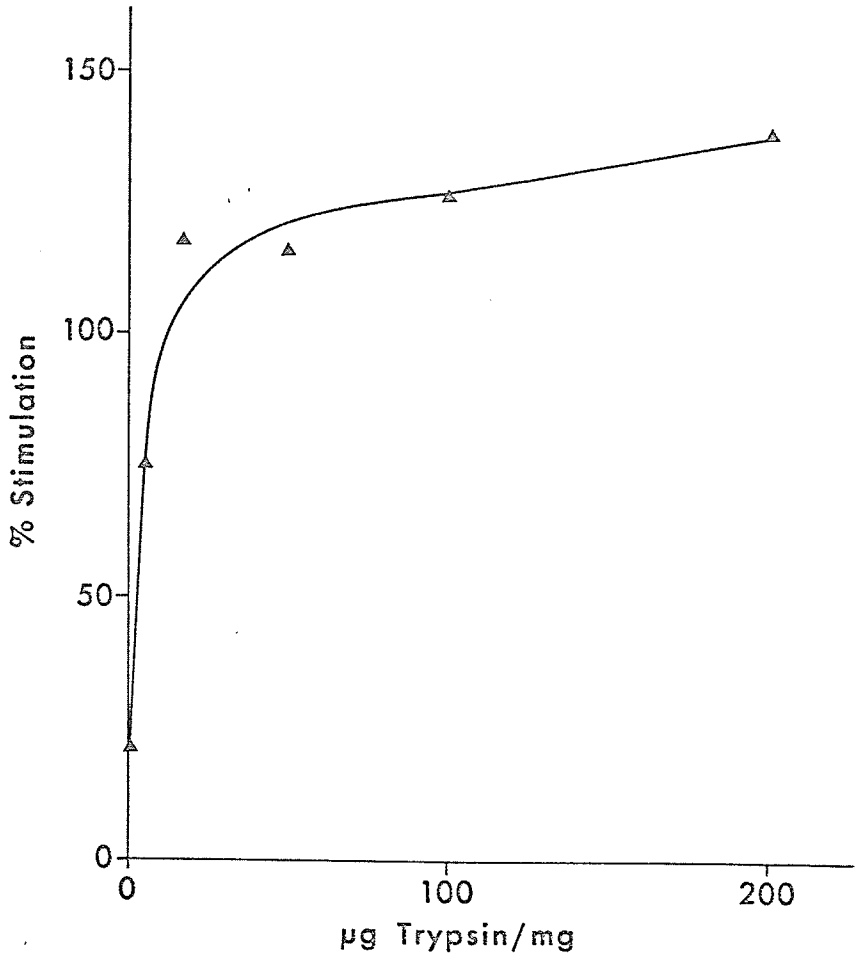

FIGURE 4 Effect of trypsin on 5'-nucleotidase in fraction HL. The assay system was carried out with 50 ug membrane fraction and the protease reaction stopped with 250 ug trypsin as described in Methods. Each value is an average of 3 experiments.

be susceptible to protease regardless of the sidedness of the vesicles.

The time and concentration dependence of the trypsin activation of 5'-nucleotidase in fraction HL is shown in Figures 3 and 4. At 10 ug trypsin per mg membrane protein, near maximal activation occurred. Also, consistent with earlier suggested ectoprotein properties (4-8) in right side-out vesicles is the observation that stimulation (at 200 ug trypsin per mg membrane protein) of 5'-nucleotidase was reached with extreme rapidity (Fig. 3). Proteolytic cleavage might have stripped the enzyme from the membrane thereby solubilizing it. However, the experiments summarized in Table 5 demonstrate that after the trypsin treatment of fraction HL no activity was detected in the supernatant (40,000xg). In addition it was also found that after preincubation at 37^{o} C, the enzyme was somewhat labile as a part of its activity in both fractions was lost after centrifugation. To investigate further the stimulatory effect of trypsin on 5'-nucleotidase in fraction HL, the kinetics of the enzyme was studied in the presence of this proteolytic enzyme. No changes in K_m for AMP after proteolytic action was observed. This means that the activation of 5'-nucleotidase by trypsin cannot be explained by an increase in affinity for AMP. In view of the observations that the enzyme in fraction S had a low affinity for AMP and its competitive inhibitor ATP, and was unaffected by trypsin or neuraminidase treatment, it seemed very likely that 5'-nucleotidase is located at the inside of these vesicles. Considering this possibility, it was decided to study the relative proportion of inside-out vesicles present in the two membrane preparations.

TABLE 5

EFFECT OF PRETREATMENT OF MEMBRANES WITH TRYPSIN ON 5'-NUCLEOTIDASE IN SOLUBLE AND PARTICULATE FRACTION.

Membrane protein (400 ug) was incubated with 0.4 mg trypsin in trypsin buffer. Protease reaction was stopped with 1.2 mg of trypsin inhibitor. Trypsin treatment was carried out as described in Methods.

		Fraction HL		Fraction S	
		Control	Trypsin	Control	Trypsin
		nmol/min/mg		nmol/min/mg	
Direct after incubation	A.	58.8	148.5	188.8	205.8
	B.	61.3	153.0	191.6	208.8
Supernatant	A.	0	0	2.1	3.1
	B.	0	0	2.7	3.9
Pellets	A.	55.3	134.8	163.0	176.1
	B.	53.9	139.6	168.2	171.9

D. Vectorial Properties of Membrane Bound Enzymes

As seen earlier the specific activity of Na^+-K^+-ATPase in both fractions was high (Table 3), however, Table 6 shows that the stimulation of ATPase produced by using optimal concentrations of Na^+ plus K^+ in fraction S was inhibited by 17% in the presence of 2mM ouabain which produced 75.6% inhibition in fraction HL. Recently, it was shown that the sensitivity to ouabain can be influenced by the vesicular orientation (24, 25) and since the ouabain binding site is located on the outer surface of the membrane the results in Table 6 seem to suggest that fraction S in comparison to fraction HL contains more inside-out vesicles. This is supported by the ratios of Na^+-K^+-ATPase to K^+-pNPPase which are significantly different in both preparations. In solubilized highly purified Na^+-K^+-ATPase preparations, the Na^+-K^+ stimulated ATP hydrolysis is about 6-10 times higher than the K^+-pNPPase activity (24). Therefore, the value 8.8 obtained for fraction HL (Table 6) is well within that range whereas the Na^+-K^+-ATPase to K^+-pNPPase ratio in fraction S was very high.

Many investigators have demonstrated that an ATP dependent Ca^{2+} binding which might be involved in myocardial Ca^{2+} extrusion appear to exist in the sarcolemma (26, 27). Accordingly, the right-sided out membrane vesicles should not exhibit any ATP dependent Ca^{2+} binding. The large difference in ATP dependent Ca^{2+} binding found in fractions HL and S (Table 6) tends to give additional support to the proposal that fraction S contains a relatively large proportion of inside-out vesicles. Lüllman and Peters (28) have also described a membrane preparation which is quite similar in properties to the fraction S. Wollenberger and Will (29) using the essentially the same preparation failed to observe the stimulation of adenylate cyclase activity by

TABLE 6

PROPERTIES OF TWO DIFFERENT MEMBRANE FRACTIONS FROM RAT VENTRICLES.

The assay systems were the same as in Methods. The absolute activity of Na^+-K^+-ATPase in both membrane fractions has been mentioned in Table 3. For ATP dependent Ca^{2+} binding, 50 ug membrane protein was incubated in 0.5 ml buffer. Values given are the means ± S.E.M. of 4 experiments.

MEMBRANE FRACTION	K^+-pNPPase (umol/h/mg)	$\frac{Na^+\text{-}K^+\text{-ATPase}}{K^+\text{-pNPPase}}$	Ouabain inhibition of Na^+-K^+-ATPase (%)	ATP dependent Ca^{2+} binding (nmol/mg protein/5 min)
Fraction HL	2.1 ± 0.1	8.8 ± 0.6	75.6 ± 7.0	2.6 ± 0.2
Fraction S	1.3 ± 0.2*	19.2 ± 2.7*	17.4 ± 5.4*	9.5 ± 1.7

* Values obtained for fraction S differed significantly ($p < 0.01$) from those obtained for fraction HL.

epinephrine. Results from our laboratory indicate no stimulation in fraction S whereas there was a 20% stimulation (by 100 uM epinephrine) of the adenylate cyclase present in fraction HL. The catecholamine receptors are believed to be situated at the external surface of the sarcolemma and thus may not be readily accessible in fraction S due to intravesicular localization.

E. Effect of Detergent on the Properties of 5'-Nucleotidase

Disruption of the integrity of the membrane bilayer to render the vesicles permeable to ions as well as phosphorylated intermediates can be induced by detergents. In the present study, up to 2% Triton X-100 was used in the 5'-nucleotidase assay in the presence of extremely low amounts of membrane protein (10 ug). Under such conditions, K^+-pNPPase in both membrane fractions was completely destroyed. However, it was found that 5'-nucleotidase in fraction HL increased by 60% in the presence of 0.5% Triton X-100, whereas no effect was observed in fraction S (Fig. 5). Further, the specific activity of 5'-nucleotidase at 200 uM relative to 80 uM AMP in the presence of the same concentration of detergent did not change significantly in both membrane fractions (Fig. 6) thus indicating that the affinity of the enzyme for substrate was unaffected; i.e. the activity of 5'-nucleotidase in the presence of 200 uM AMP was compared to its activity in the presence of 80 uM AMP in order to observe the effect of 2% Triton X-100 on its kinetic behavior in the two sarcolemmal preparations. Since there was little or no change in the activity of the nucleotidase, it is very likely that there was also no change in its kinetic properties in either fraction HL or S. These findings appear to demonstrate that the low affinity of 5'-nucleotidase

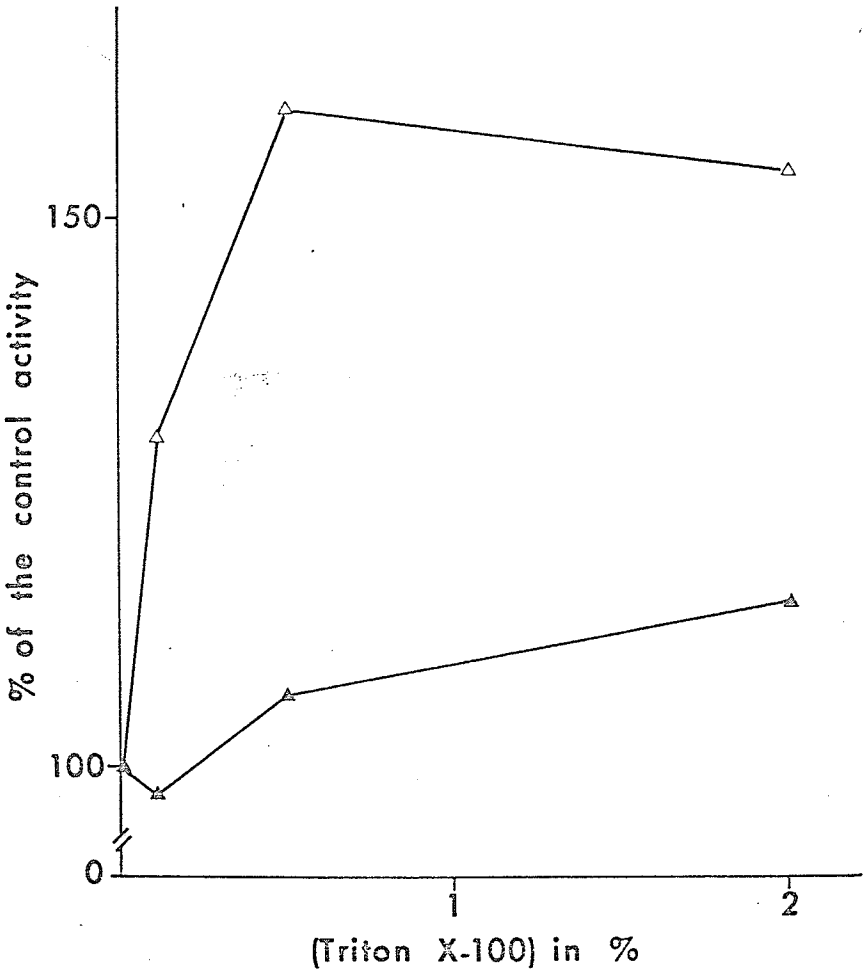

FIGURE 5 Effect of Triton X-100 on the activity of 5'-nucleotidase in fractions HL (△—△) and S (▲—▲). The assay was performed with 10 ug membrane protein as described in Methods except that various amounts of Triton X-100 were employed. The results are typical of 3 experiments.

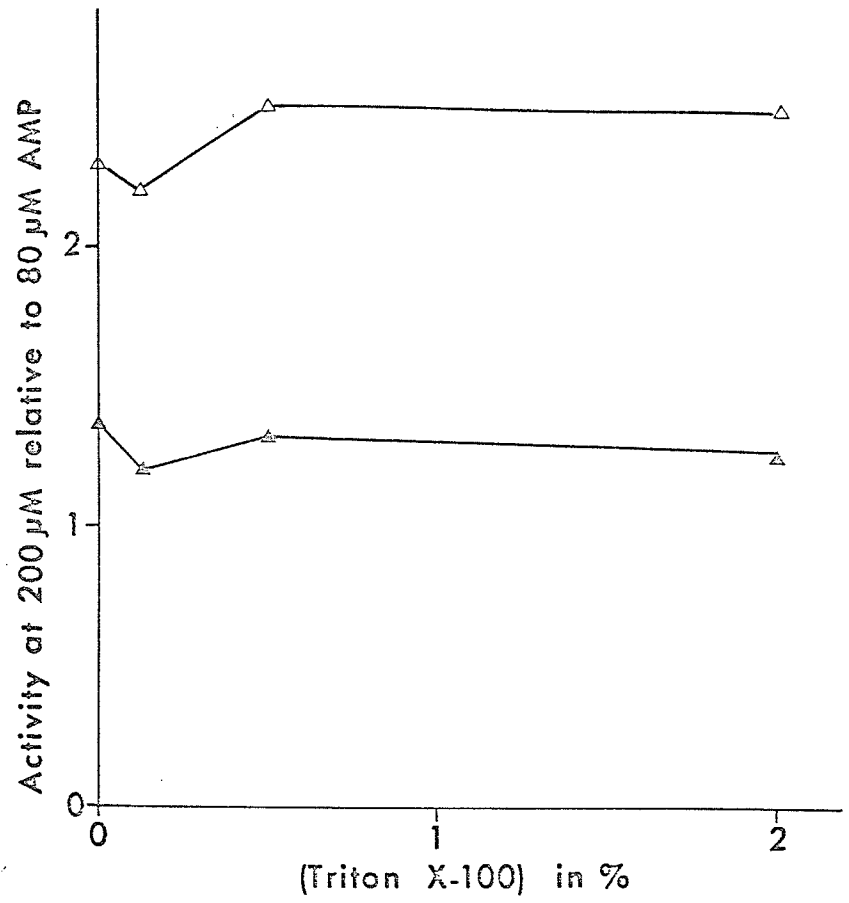

FIGURE 6 Effect of Triton X-100 on the kinetics of 5'-nucleotidase in fractions HL (▲——▲) and S (△——△). The amount of membrane protein (10 ug) used was the same as that used to produce the results presented in Figure 5. The assay system was the same as described in Methods and Figure 5. Results are typical of 3 experiments.

for AMP in fraction S cannot be explained by having the catalytic site exposed to the interior of the membrane vesicles. Furthermore, at 2% Triton X-100 the membranes should be completely solubilized and thus there should not be any difference in the activity of these preparations. Since this was not found to be the case, it is likely that the observed differences in both membrane preparations are due to some inherent differences in the properties of the enzyme. A similar conclusion has been made by Doss et al (97) while working on 5'-nucleotidase in mammary glands. They observed a difference in the kinetic behavior of the enzyme in two different plasma membrane fractions (F_1 & F_2) obtained by floatation of microsomes on a discontinuous sucrose density gradient. Whereas solubilization of F_1 with 1% Triton X-100 resulted in a conversion of essentially all of the nucleotidase from a high to a low K_m form exhibited in fraction F_2, there was no change in the K_m of the enzyme in fraction, F_2. In view of these results they concluded that there is one type of enzyme whose properties are dependent on its association with the membrane; hence, as indicated by our results, the side of the membrane on which the nucleotidase is located, at the outside or inside, might be a factor. Since we observed no change in the kinetic behavior of the enzyme after treating the membrane with 2% Triton X-100, it might be that this concentration of detergent was not strong enough to solubilize the nucleotidase in cardiac tissue.

F. Concanavalin A Inhibition, -SH Group Dependency pH Optimum and Heat Stability

Previous studies have shown that 5'-nucleotidase in plasma membranes obtained from liver (30, 31), mammary gland (32) and cell

cultures (32, 33) was inhibited by concanavalin A (Con A). Specific binding to carbohydrate structures rather than non-specific protein - protein interaction is involved, because the inhibition (or binding) can be relieved by α - methylmannoside. Little and Widnell (31) found differences in Con A sensitivity in different membrane fractions obtained from rat liver but it was established that this was due to the location of 5'-nucleotidase in the vesicles of the fractions. Since Con A is a plant lectin with a molecular weight of about 50,000 (34), the passage across intact membrane vesicles might be blocked. In either fraction (HL or S), Con A caused almost complete inhibition of 5'-nucleotidase (Fig. 7) and the amount of this lectin required for half maximal inhibition appeared to be the same (75 ug Con A/mg membrane protein) for both preparations. Inclusion of 10 mM α - methylmannoside prevented the inhibitory effect of Con A in the two fractions. The results in Fig. 7 show that nearly 95% of enzyme activity could be blocked by 2 uM Con A indicating that the phosphohydrolase estimated is predominantly, if not exclusively, 5'-nucleotidase. Moreover, the contribution by non-specific phosphatase to AMP hydrolysis is unlikely because of the presence of 2 mM dicyclohexylammonium p-nitrophenyl phosphate during the assay. When the data in Fig. 7 were analysed for co-operativity, the Hill coefficient for fractions HL and S were 1.1 and 2.2 respectively. This indicates that the interaction of Con A with surface glycoproteins to induce 5'-nucleotidase inhibition involves at least two co-operative binding sites in fraction S. Furthermore, a consistent discontinuity was observed in the shape of the curve for fraction HL at low (0.05 uM) Con A concentration.

Preincubation of membranes for 5 minutes at 37^{o} C with

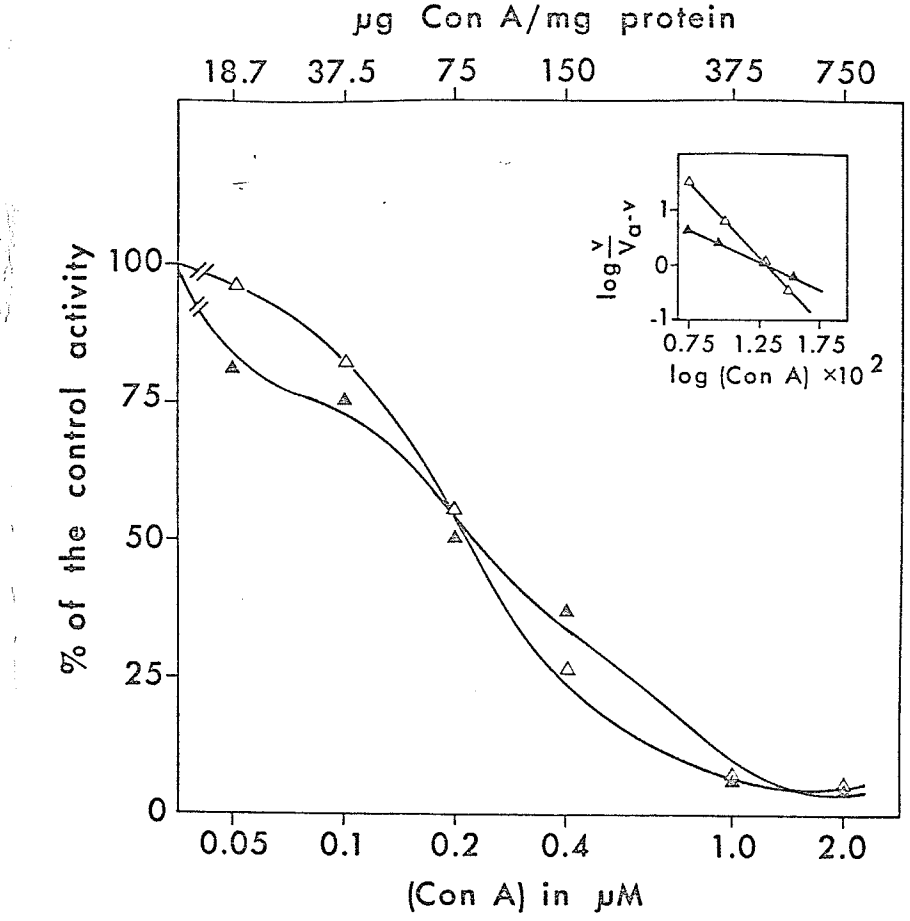

FIGURE 7 Effect of concanavalin A on the activity of 5'-nucleotidase in fractions HL (▲—▲) and S (△—△). The assay was performed with 30 ug of either membrane fraction as described in Methods except that various concentrations of concanavalin A (Con A) were employed. The inset shows a Hill plot for the Con A inhibition of 5'-nucleotidase. The results are the average of 2 experiments.

concentrations of up to 0.1 mM p - hydroxymercurybenzoate did not produce any significant effect on the enzyme in either preparation (Table 7). This demonstrates that the activity of 5'-nucleotidase in both preparations is not dependent on free -SH groups (Table 7). In another series of experiments, it was observed that the enzyme was active over a broad pH range with optimal activity at pH 7.0 - 8.0 for both fractions were very similar (Fig. 8). Furthermore, 5'-nucleotidase activity was found to diminish gradually on heating at 60^{o} C (Fig. 9), although about 25% of the total activity seemed to be stable. The enzyme in fraction S appeared to lose activity more rapidly than that in fraction HL.

2. Effect of Drugs on Heart Sarcolemmal 5'-Nucleotidase

Figs. 10 and 11 present dose response curves for caffeine and theophylline in fractions HL and S respectively. Both drugs inhibited 5'-nucleotidase but the antagonistic effect of theophylline was greater than that of caffeine; this difference was clearly shown at higher drug concentrations. Significant inhibition was achieved in both preparations with methylxanthine concentrations as low as 0.03 mM. However, the enzymatic inhibition was less than 20% in fraction HL for concentrations of either drug between 0.03 mM and 0.3 mM whereas the same concentrations of caffeine inhibited the enzyme by less than 20% in fraction S and a 0.3 mM theophylline exerted a 26% inhibition in this sarcolemmal preparation. A 1 mM concentration of either caffeine or theophylline inhibited the enzyme in fraction HL by 27% and 33% respectively while in fraction S the same concentration of either methylxanthine produced 28% and 42% of enzymatic inhibition. Similarly, at a 3 mM concentration of either drug, the enzymatic inhibition was

TABLE 7

The effect of p-hydroxymercurybenzoate on heart sarcolemma 5'-nucleotidase. Values given are the means ± S.E.M. of 3 experiments.

p-hydroxymercurybenzoate	5'-Nucleotidase	
	Fraction HL	Fraction S
	% Control	
10^{-7} M	94 ± 7.4	98 ± 3.6
10^{-6} M	101 ± 12.6	108 ± 11.7
10^{-5} M	102 ± 14.9	101 ± 8.6
10^{-4} M	98 ± 5.7	106 ± 7.2

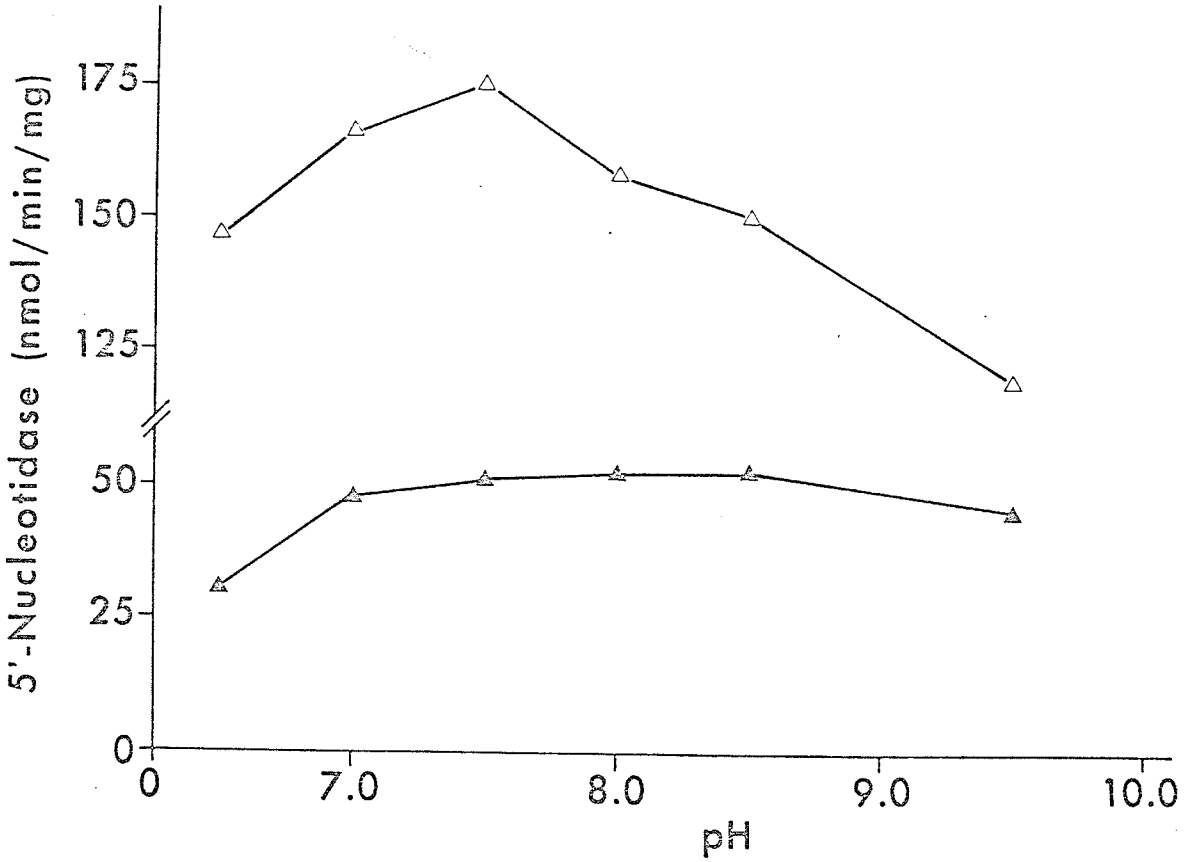

FIGURE 8 Effect of pH on the activity of 5'-nucleotidase in fractions HL (▲—▲) and S (△—△). The assay was performed with 20 ug of either membrane fraction as described in Methods. The results are typical of 3 experiments.

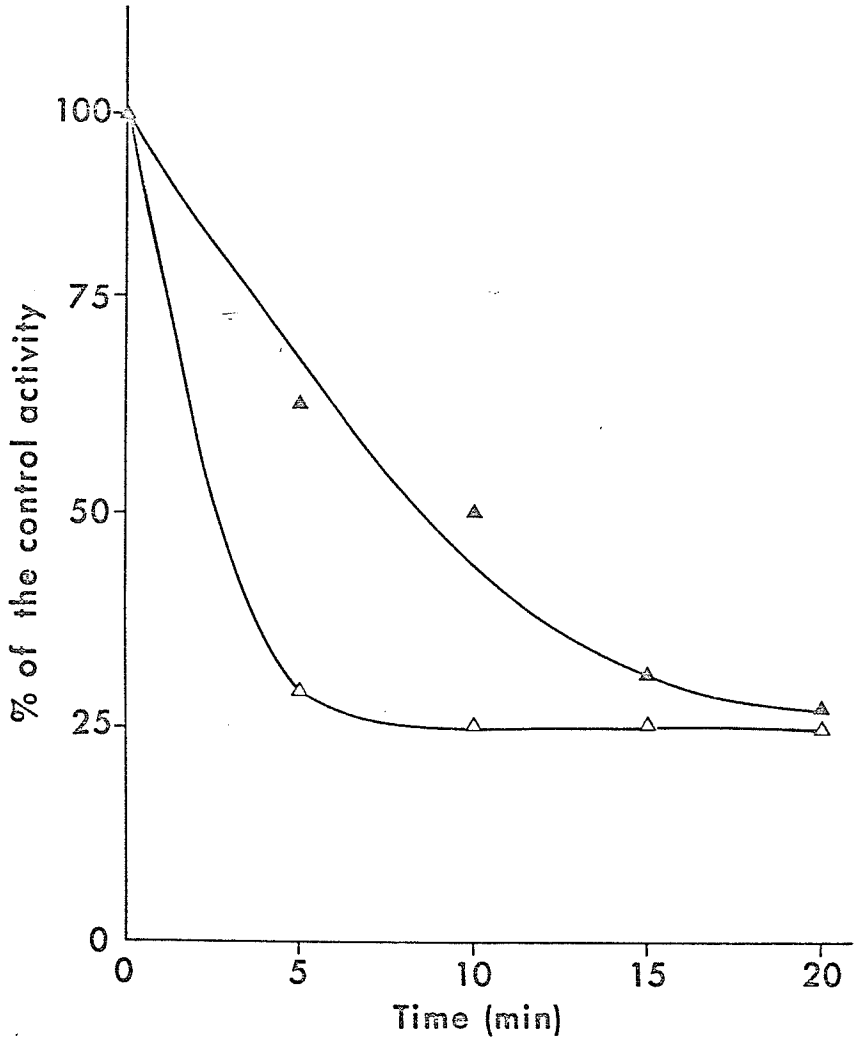

FIGURE 9 Heat stability of 5'-nucleotidase in fractions HL (▲—▲) and S (△—△). The assay was carried out with 20 ug membrane fractions as outlined in Methods except the preincubation temperature was 60°C. The results are typical of 3 experiments.

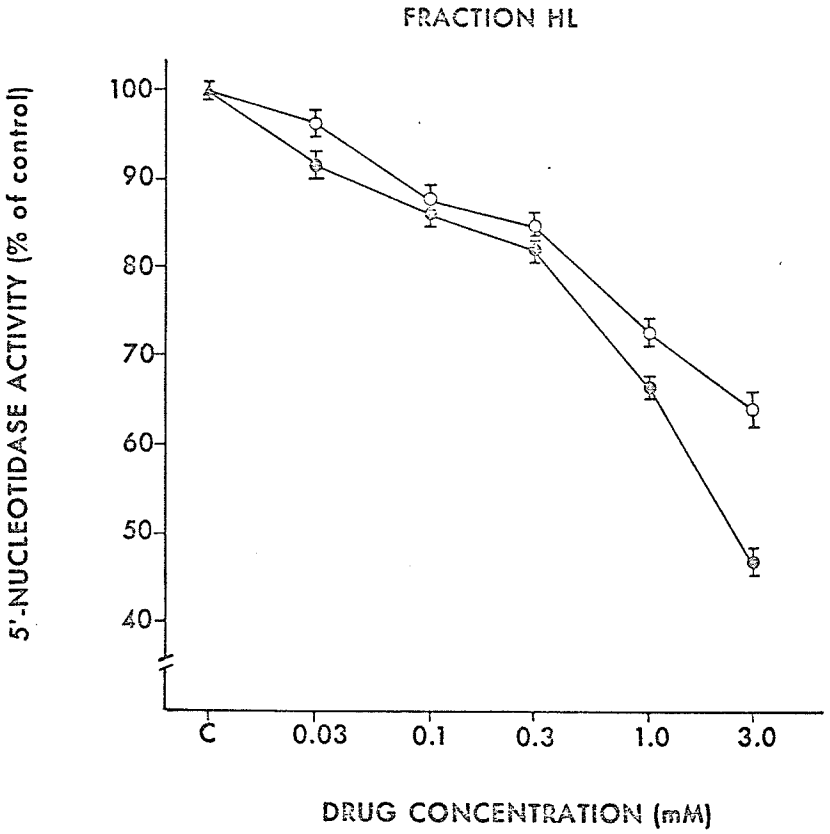

FIGURE 10 Dose response relation for the inhibition by caffeine and theophylline of 5'-nucleotidase in fraction HL. Control (▲), caffeine (O) and theophylline (●). The assay system was performed with 60 ug membrane protein and was the same as described in Methods except that different concentrations of the methylxanthines were employed. Results shown are the means ± S.E.M. of 4 experiments.

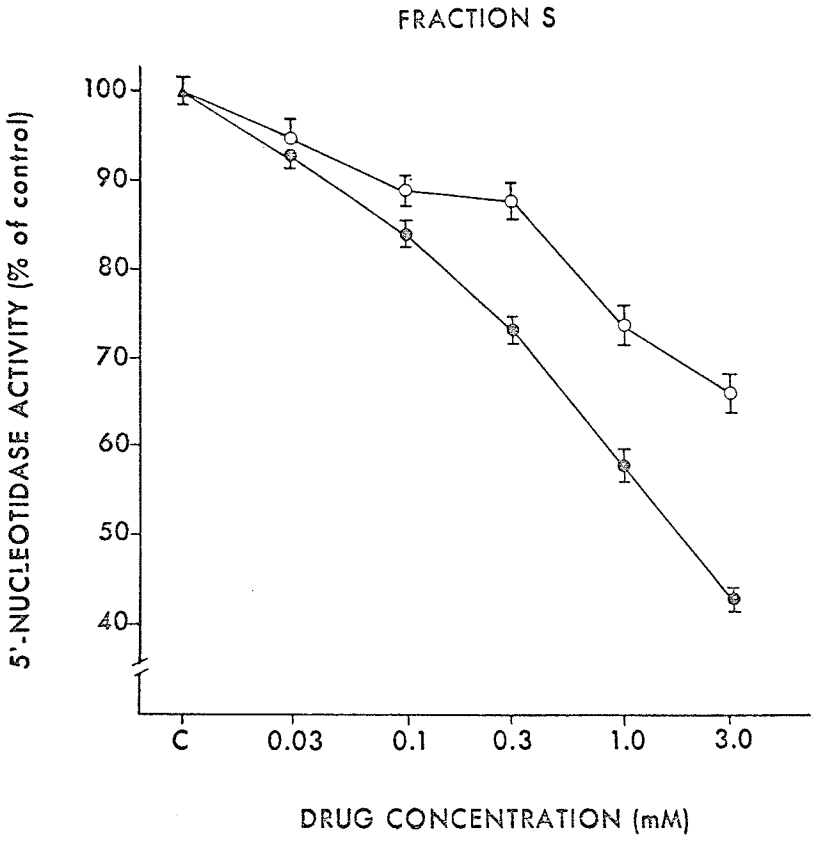

FIGURE 11 Dose response relation for the inhibition by caffeine and theophylline of 5'-nucleotidase in fraction S.
Control (▲), caffeine (○) and theophylline (●). The amount of membrane protein used and the assay system were the same as described for Figure 10. Results shown are the means ± S.E.M. of 4 experiments.

35% and 53% in fraction HL, and 36% and 57% in fraction S. Neither dipyridamole nor papaverine regardless of the concentration, appeared to affect 5'-nucleotidase in the sarcolemmal preparations (Figs. 12 and 13). 0.5mM was the highest concentration of either drug used in this study as higher concentrations lead to their precipitation in the assay mixture. At this concentration however, both methylxanthines inhibited the nucleotidase in the two sarcolemmal preparations. The time course study also show the antagonistic effect of two methylxanthines on 5'-nucleotidase in both sarcolemmal preparations (Figs. 14 and 15). However, the pattern of enzymatic activity in both fractions was virtually unchanged in the presence of these two drugs, i.e. there was no marked deviation in the inhibition of the enzyme activity over a period of 30 minutes in the presence of caffeine and theophylline. This study also shows that the inhibition of the enzyme by theophylline was greatest after a 10 minute incubation period which was also the time period employed for studying the effect of the two methylxanthines on the nucleotidase in both sarcolemmal preparations. After a 30 minute incubation period with these two drugs however, the inhibition by caffeine appeared to decrease in fraction HL in contrast to a reduction in inhibition by theophylline in fraction S. This finding seem to suggest that a dose-response relationship between 5'-nucleotidase and these two methylxanthines in both fractions (HL and S) after 30 minutes of incubation will be different from that observed after a ten minute incubation period.

As shown in Figs. 16 and 17, 5'-nucleotidase in fraction HL had an apparent K_m for AMP of 0.04mM while the K_m in the presence of 1 mM caffeine and 1 mM theophylline were 0.08mM and 0.11mM respectively. The K_m for the enzyme in fraction S was 0.133mM in contrast to a K_m of

0.17mM and 0.20mM in the presence of 1 mM concentrations of caffeine and theophylline respectively. The inhibition was of the competitive type in both sarcolemmal fractions.

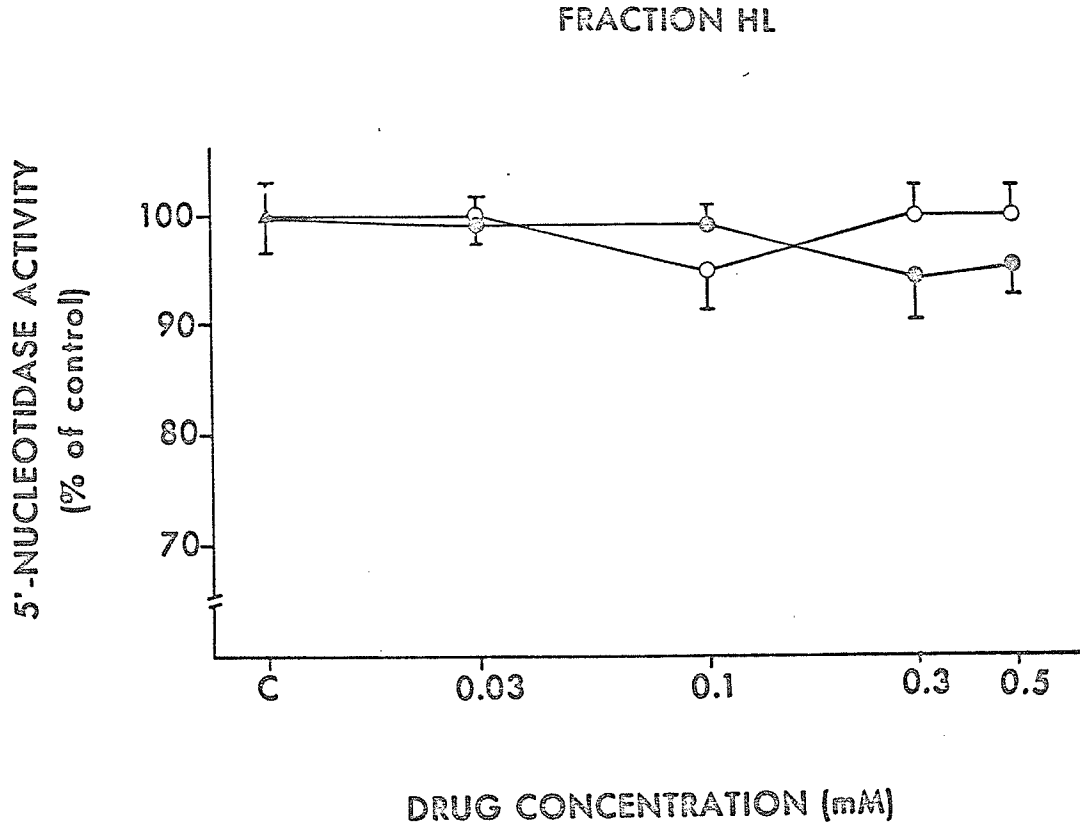

FIGURE 12 Dose response relation between 5'-nucleotidase in fraction HL and two coronary vasodilatory drugs: (dipyridamole and papaverine). Control (▲), dipyridamole (●), and papaverine (O). The assay system was the same as described in Methods except that various concentrations of vasodilators were employed. The results are the means ± S.E.M. of 3 experiments.

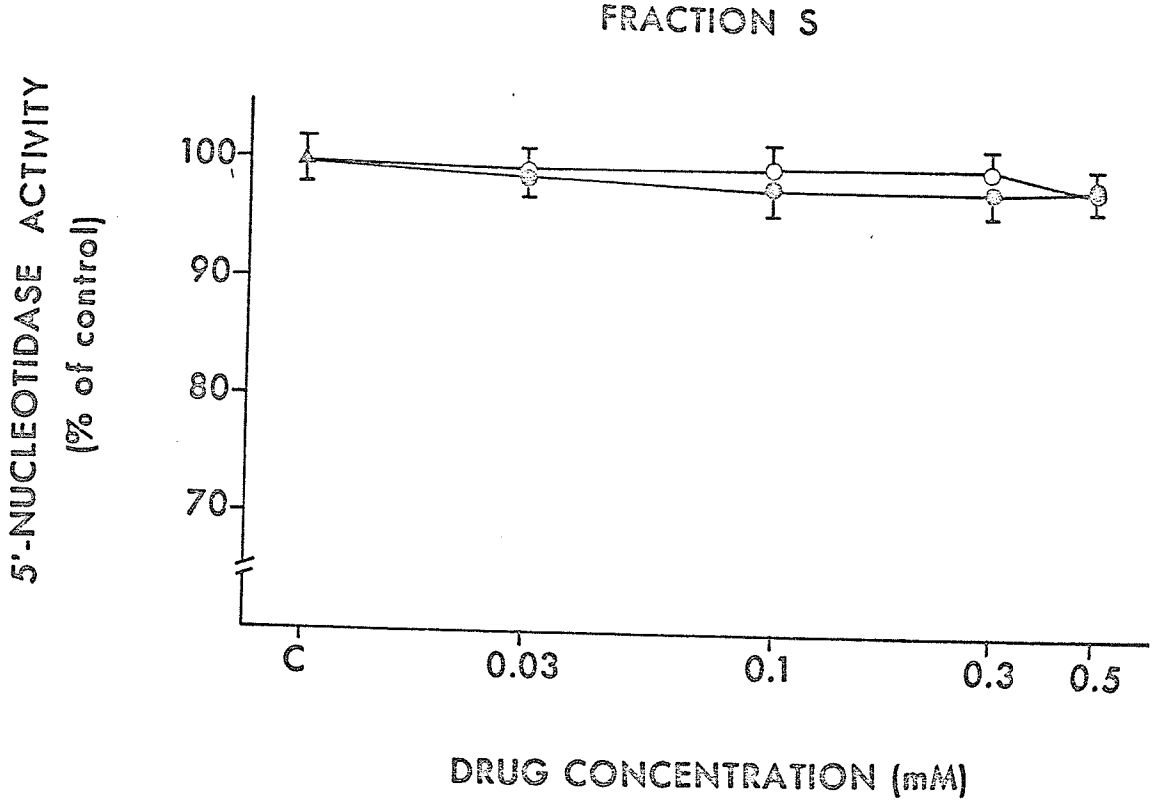

FIGURE 13 Dose response relation between 5'-nucleotidase in fraction S and two coronary vasodilatory drugs (dipyridamole and papaverine). Control (▲), dipyridamole (●) and papaverine (○). The amount of membrane fraction (80 ug) and the assay system were the same as described for Figure 12. Results are the means ± S.E.M. of 3 experiments.

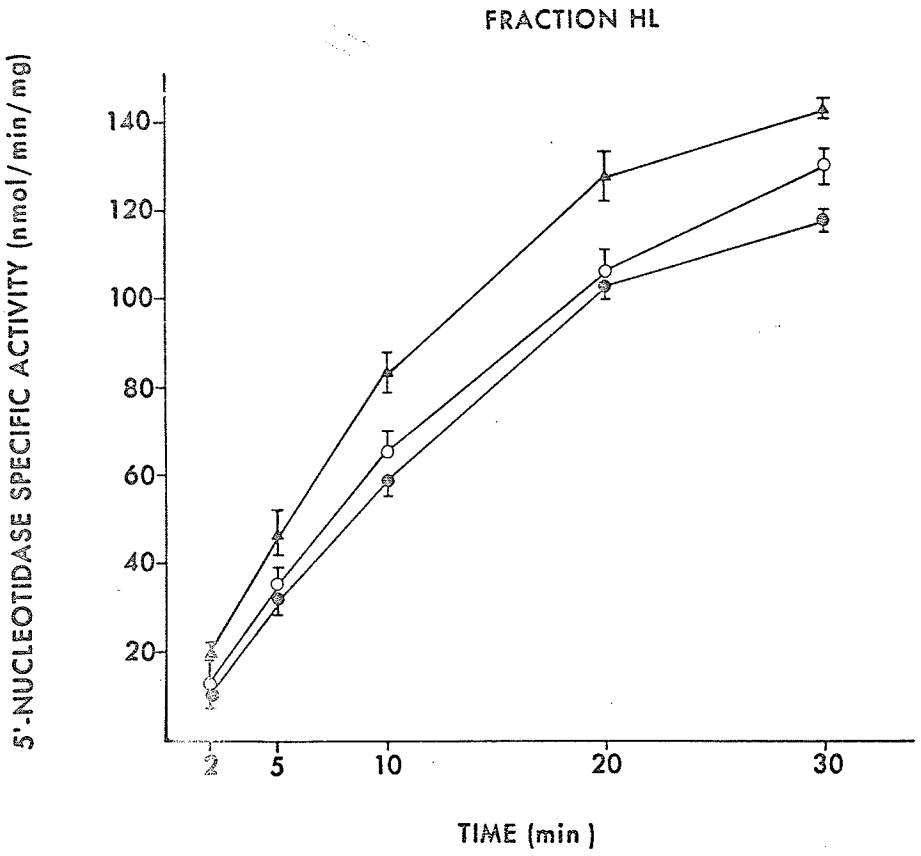

FIGURE 14 Time course study of the effect of caffeine (1mM) and theophylline (1mM) on the activity of 5'-nucleotidase in fraction HL. Control (▲), caffeine (○) and theophylline (●). The assay system was performed with 65 ug membrane protein as outlined in Methods except that 1mM caffeine and 1mM theophylline were employed at different intervals of time. The results are the means ± S.E.M. of 3 experiments.

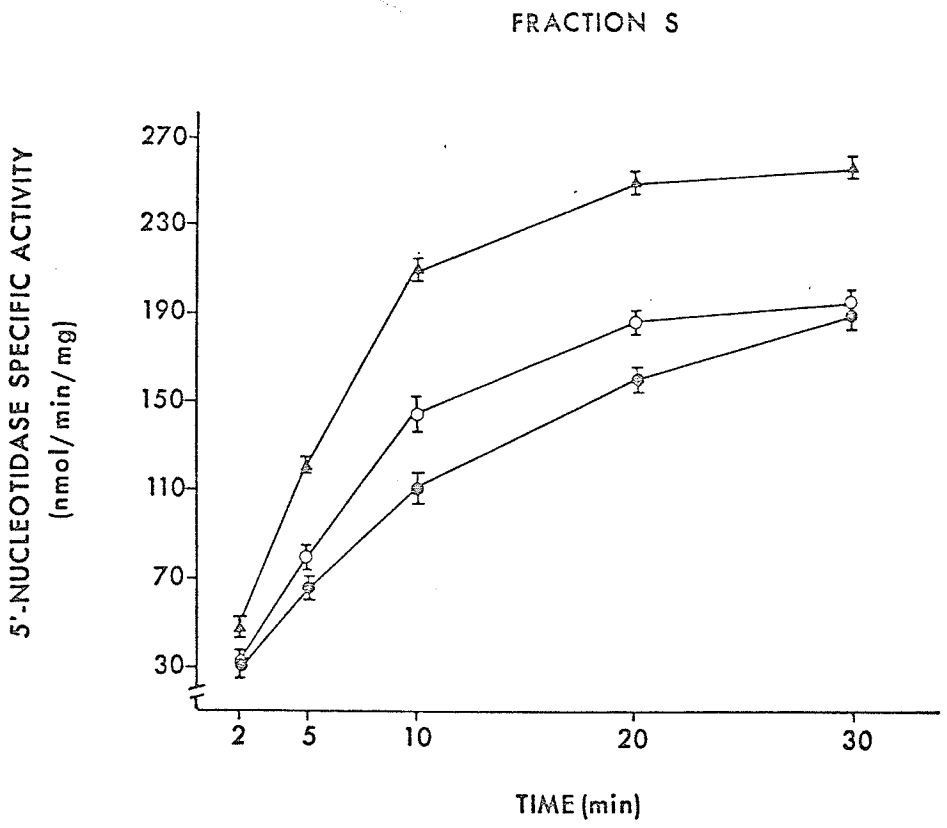

FIGURE 15 Time course study of the effect of caffeine (1mM) and theophylline (1mM) on the activity of 5'-nucleotidase in fraction S. Control (▲), caffeine (○) and theophylline (●). The assay system, including the amount of membrane protein used, was the same as described in Figure 14. The results are the means ± S.E.M. of 3 experiments.

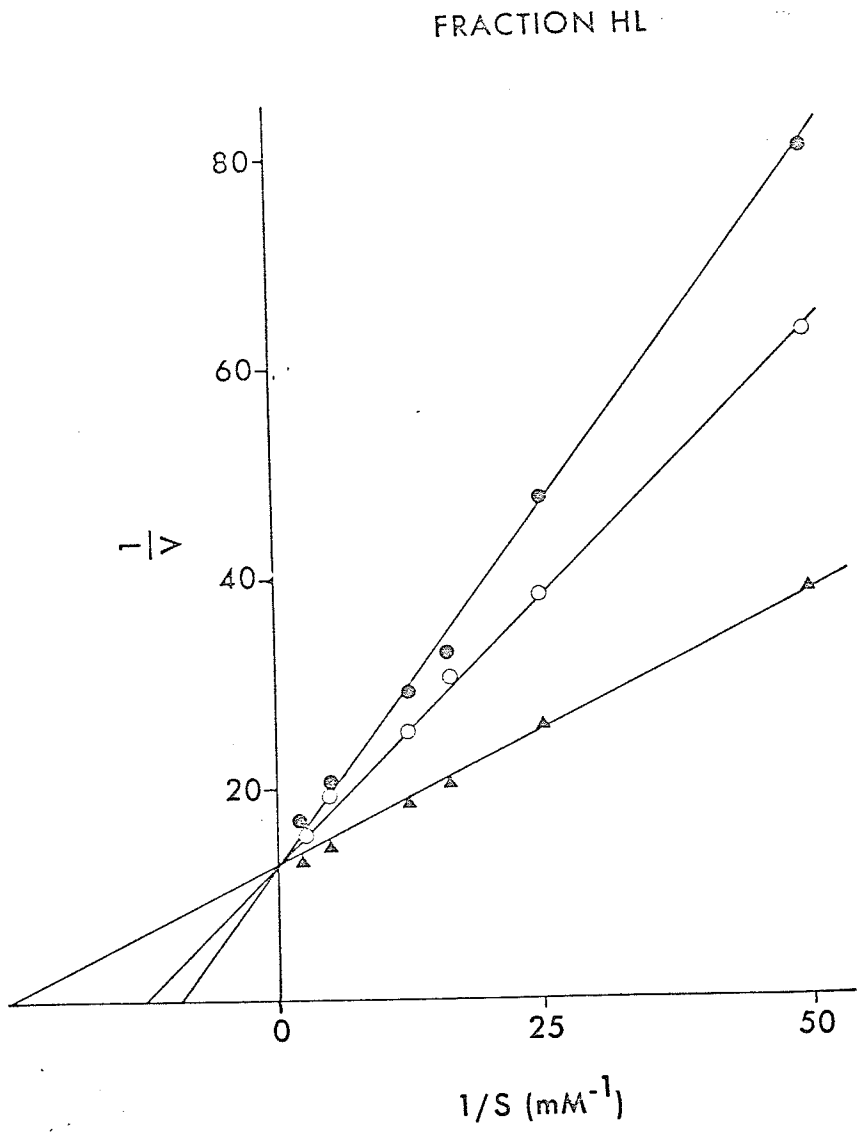

FIGURE 16 Inhibition of cardiac sarcolemmal 5'-nucleotidase of the rat ventricle by caffeine and theophylline in fraction HL. Control (▲), caffeine (○) and theophylline (●). The assay was performed with 75 ug membrane protein as outlined in Methods except that 1mM caffeine and 1mM theophylline were employed. V, nmol of adenosine formation. min^{-1}. mg^{-1}. S, AMP. Results are the means of 2 experiments.

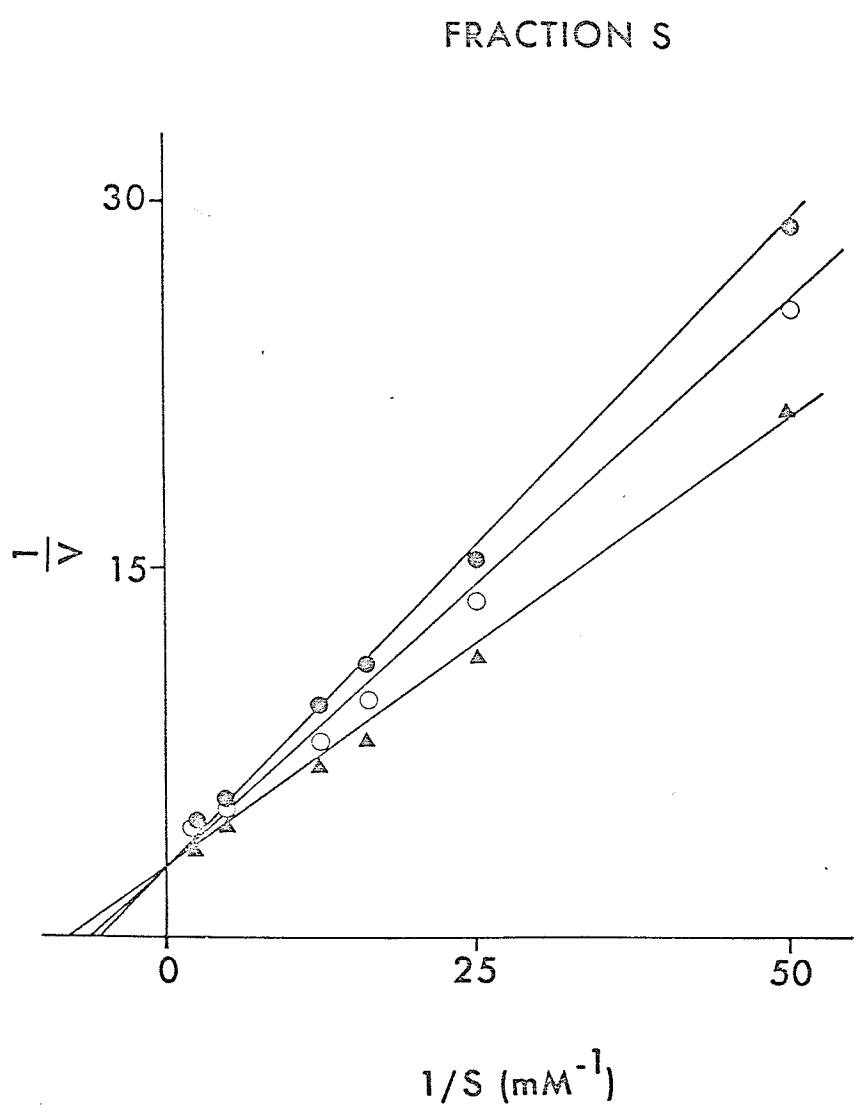

FIGURE 17 Inhibition of cardiac sarcolemmal 5'-nucleotidase of the rat ventricle by caffeine and theophylline in fraction S. Control (Δ), caffeine, 1mM (O) and theophylline, 1mM (●). The assay system and amount of membrane protein used were the same as described in Figure 16. V, nmol of adenosine formation. min^{-1}. mg^{-1}. S, AMP. Results are the means of 2 experiments.

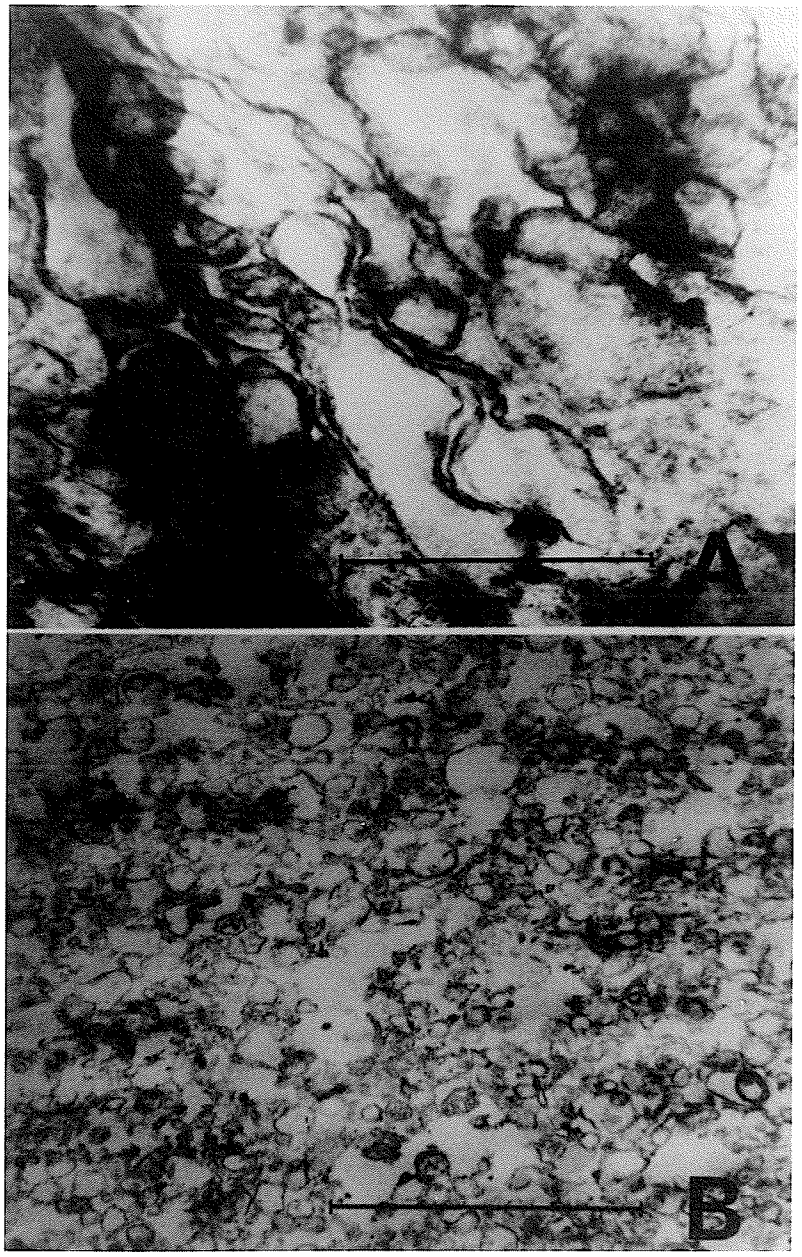

FIGURE 18. Upper panel (A): Electron micrograph of a typical sarcolemmal fraction obtained by hypotonic shock-LiBr method (Fraction HL). Black bar indicates one micron.

Lower panel (B): Electron micrograph of a typical sarcolemmal fraction obtained by sucrose density gradient method (Fraction S). Black bar indicates one micron.

DISCUSSION

A. Characterization of Heart Sarcolemmal 5'-Nucleotidase

The results reported in this study indicate that a major portion of the total 5'-nucleotidase activity in the rat heart homogenate is present in the sarcolemmal fraction. This is consistent with the view that this enzyme is mainly located at the cell membrane. Although other particulate fractions also contained small amount of the total activity it is difficult to determine on the basis of available information whether or not this enzyme activity is due to redistribution during the process of isolation. While this point needs to be settled by future studies, the experiment described here reveal a marked inhibitory effect of ATP on the sarcolemmal 5'-nucleotidase. In both preparations, the inhibition was of the competitive type. Baer et al (35) working with a solubilize cardiac preparation also found this type of competitive inhibition in the presence of ATP. The K_m (35 uM) for AMP in fraction HL was close to that found for the solubilized enzyme whereas in fraction S it was higher. Although Nakamura (36) observed a higher K_m for substrate in the purified vesicular structures of liver 5'-nucleotidase compared to the solubilized form, and Doss et al (97), using 1% Triton X-100 solubilized the enzyme in mammary gland thereby changing its kinetic behavior, we were not able to change the affinity for AMP by adding high concentrations of Triton X-100 in the assay medium. This may be due to the nature or tissue source of the membrane vesicles employed in this study. It might be too, that 2% Triton X-100 was not strong enough to solubilize the enzyme in cardiac tissue.

The most interesting finding to emerge from this study was a clear-cut difference in the response of 5'-nucleotidase in fractions HL and S to trypsin and neuraminidase digestion of the membranes. These

results seem to suggest that at least in fraction HL the active site of the enzyme might be mainly exposed to the external medium. In two reports about enzymatic digestion of liver plasma membranes and the subsequent effect on 5'-nucleotidase it was shown that neuraminidase did not affect the enzyme even after the removal of 70% of the membrane bound sialic acid (37). Tryptic digestion slightly increased the activity in purified liver membranes, but there was no effect on the detergent solubilized enzyme (36). In our study, tryptic and neuraminidase digestion significantly increased the activity of 5'-nucleotidase in fraction HL but had little or no effect on the enzyme in fraction S. There was no change for the affinity of AMP after the Triton X-100 and trypsin treatments. The time course and concentration dependency studies for trypsin activation of the enzyme in fraction HL seem to suggest that the protease acts very rapidly. The simplest explanation for the foregoing results obtained with fraction HL can be that 5'-nucleotidase is an outer surface constituent of the sarcolemma. The use of vectorial properties of membrane bound systems as Na^+-K^+-ATPase, adenylate cyclase, and ATP dependent Ca^{2+} binding has allowed a tentative assignment of fraction HL as containing predominantly right-side out vesicles. Further, the dense lanthanum staining of sarcolemmal suspension of fraction HL in contrast to fraction S, as revealed by electron micrographic studies also supports the suggestion of a predominance of right-side out vesicles in this fraction (Fig. 18). Lanthanum staining is specific for mucopoly-saccharide groups at the basement of the cell surface (46). Alterations in the basement membrane of the right-sided out vesicles in the fraction HL by neuraminidase may make more sites of the 5'-nucleotidase and this may explain the increased enzyme activity upon neuraminidase treatment.

There are several recent reports which tend to suggest an ectolocalization of 5'-nucleotidase (1, 4 - 8). The most convincing experiments were carried out by using antibodies raised against purified 5'-nucleotidase (6) and in isolated cells (5). The hydrolysis of AMP observed by Frick and Lowenstein (10) during their perfusion of the isolated rat heart also tends to support the ectolocalization of this enzyme.

Our modified sucrose gradient procedure resulted in significantly higher activities for adenylate cyclase and 5'-nucleotidase than those obtained by Kidwai et al (18) and Moffet et al (38). A very similar method was used by Lüllman and Peters (28) and Wollenberger and Will (29). With the aid of an extracellular marker during the perfusion and homogenization of the heart, Lüllman and Peters were able to show that their procedure produced inside-out vesicles. In the case of fraction S, our finding of a low ouabain sensitive Na^+-K^+-ATPase, high ATP dependent Ca^{2+} binding, high Na^+-K^+-ATPase specific activity relative to K^+-pNPPase, and the sensitivity of adenylate cyclase to epinephrine are all in agreement with the findings of Lüllman and Peters (28) and Wollenberger and Will (29). They identified this membrane fraction with the transverse tubular part of the sarcolemma. Electronmicroscopic examination of fixed tissue preparations carried out by Rostgaard and Behnke (3) and independently by Gordon et al (2) have shown that 5'-nucleotidase is particularly active at the T-tubule system. In view of these reports the high specific activity of 5'-nucleotidase in fraction S may be explained by assuming a predominance of T-tubules in this fraction. However, the different properties of the enzyme in this preparation as compared to those in fraction HL seem to suggest the presence of an active 5'-nucleotidase exposed to the cytoplasm in vivo.

The low affinity for substrate AMP and competitive inhibitor ATP would fit better into a possible regulation of 5'-nucleotidase by adenine nucleotides only.

Further, the difference in co-operativity of Con A inhibition of the nucleotidase in fractions HL and S seem to suggest that its properties in fraction HL are different from those in fraction S. These findings suggest that a) there is one type of enzyme whose properties are dependent on its association with the membrane and b) the enzyme can exert its action in vivo at either side of the plasma membrane. Similar evidence has been obtained by Frick and Lowenstein (10) in the perfused rat heart. Therefore, in a model of 5'-nucleotidase with an active site on either side of the plasma membrane, as hypothesized by Arch and Newsholme (10), and suggested by our data, it might be that only one of the subunits is exposed to the extracellular phase (as exemplified by fraction HL), with a multisubunit, as suggested by Carraway et al (9) exposed to the intracellular phase (exemplified by fraction S). This arrangement can explain the observed difference in co-operativity of Con A inhibition of 5'-nucleotidase in the two sarcolemmal fractions: non-co-operativity in fraction HL in contrast to co-operativity in fraction S. This speculation tends to be consistent with the observed difference in heat stability and enzyme kinetics because the assymetric membrane structure will lead to a different protein and phospholipid environment of the 5'-nucleotidase subunits.

B. Effect of Drugs on Heart Sarcolemmal 5'-Nucleotidase

In this study we have examined the relationship between cardiac sarcolemmal 5'-nucleotidase and 4 exogenous agents which have a common

action; i.e. they increase intracellular cAMP levels by directly inhibiting phosphodiesterase (55, 59, 62, 68, 71). Dipyridamole and papaverine increase coronary blood flow by decreasing coronary resistance (51, 56). It has been suggested that they exert this effect through their inhibition of phosphodiesterase (55, 59). They are more potent inhibitors than caffeine or throphylline (53, 60, 63). Another mechanism whereby dipyridamole is believed to bring about vasodilation is by increasing the extracellular adenosine levels by

a) inhibiting its uptake through the plasma membrane and

b) inhibiting its breakdown by adenosine deaminase (46, 51, 54).

Papaverine can also increase adenosine levels (54). Neither of these drugs had any effect on the activity of 5'-nucleotidase. Thus, it appears that this sarcolemmal enzyme is not directly involved in any mechanism by which both dipyridamole and papaverine might bring about coronary vasodilation.

In contrast, the actions of caffeine and theophylline on 5'-nucleotidase were completely different. Both inhibited the enzyme in the two sarcolemmal fractions (fractions HL and S) but theophylline appeared to be the more potent inhibitor. The depressant effect of caffeine seemed to be the same for the enzyme in both sarcolemmal preparations but 5'-nucleotidase in fraction S appeared to be more sensitive to theophylline than that localized in fraction HL. These results therefore suggest that adenosine production in the myocardium can be affected to a greater extent by theophylline than caffeine. The competitive type of inhibition exhibited by these two methyl-xanthines seems to indicate that they exert a strong influence on a coronary dilator mechanism involving 5'-nucleotidase. Since theophylline attenuated the vasodilatory action of exogenous adenosine and adenine

nucleotides in the myocardium (47), it can be suggested that this effect could have been partly due to the inhibition of endogenous adenosine production. Hence, it appears conceivable that under in vivo conditions the inhibition of 5'-nucleotidase can be partially responsible for any altered vasodilatory effect of endogenous adenosine in the presence of this methylxanthine. The same conclusion can be reached for any antagonistic effect of caffeine on exogenous adenine nucleotides and adenosine in the myocardium.

In addition to its coronary vasodilatory action, adenosine also inhibits the release of norepinephrine in the heart (56) and antagonizes the catecholamine-induced stimulation of cardiac adenylate cyclase (52, 64). Since the level of this metabolite rises concomitantly with an increase in catecholamine release (53), it has been suggested that adenosine can act as a negative feedback inhibitor of myocardial adenylate cyclase thereby preventing sympathetic over-stimulation (64). But caffeine and theophylline attenuated this effect of adenosine on the heart (50, 64). Theophylline also increased catecholamine secretion from the adrenal medulla (62), and methyl-xanthines in general inhibit the extraneuronal inactivation of catecholamines (58). Thus, the inhibition of 5'-nucleotidase appears to be involved in a mechanism by which methylxanthines can increase extraneuronal catecholamine levels and potentiate their effect on the myocardium. It can also be concluded that by inhibiting 5'-nucleotidase caffeine and theophylline will be affecting an important feedback mechanism which limits the inotropic and metabolic effects of catecholamines in the myocardium.

CONCLUSION

In this study, the characteristics of 5'-nucleotidase in heart sarcolemma was investigated. From the data obtained in the study, the following conclusions are drawn:

1) 5'-Nucleotidase is present in heart sarcolemma and its properties appear to depend on its association with the membrane.

2) Active sites of this enzyme appear to exist on both the extra and intracellular surfaces of the plasma membrane.

3) The enzyme in general is active over a wide pH range.

4) 5'-Nucleotidase, regardless of its location does not appear to depend on free (-SH) groups for its activity.

5) The enzyme is inhibited by ATP; the inhibition is of the competitive type.

6) Dipyridamole and papaverine do not appear to affect the activity of 5'-nucleotidase.

7) Caffeine and theophylline inhibit the enzyme; the inhibition is of the competitive type.

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