Adenylate metabolism and adenosine formation in the heart

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NAKATSU, KANJI, AND GEORGE I. DRUMMOND. Adenylate metabolism and adenosine formation in the heart. Am. J. Physiol. 223(5): 1119–1127. 1972. Adenylate metabolism in ventricular tissue was studied with respect to the possibility that adenosine, a catabolic product of 5'-AMP, may mediate autoregulation of coronary blood flow. The levels of 5'-nucleotidase (EC 3.1.3.5), adenylate deaminase (EC 3.5.4.6), and adenylosuccinate lyase (EC 2.7.4.3) were determined in homogenates of rat, rabbit, dog, pigeon, and turtle hearts. The absence of a specific mechanism for adenosine formation in cardiac muscle tissues and the presence of substantial 5'-nucleotidase activity in mammalian ventricle suggests that adenosine may be involved in autoregulation only in mammals. Histochemical studies revealed that 5'-nucleotidase activity was located predominantly in endothelial cells of capillaries. Thus, adenosine is involved in control of coronary blood flow, its source being capillary endothelial cells rather than cardiac muscle cells. Membrane-bound 5'-nucleotidase was isolated from rat hearts, solubilized with deoxycholate, and certain properties examined that could be involved in regulation of adenosine formation. The enzyme did not have an absolute requirement for divalent cation but was stimulated by Mg2+, the effect of this cation being to increase V_max. The enzyme possessed a broad substrate specificity, hydrolyzing a number of ribo- and deoxyribonucleoside monophosphates. 5'-UMP was the preferred substrate. The enzyme was inhibited by ATP, ADP, and orthophosphate. These properties and the intense activity present in endothelial cells are discussed in relation to the adenosine hypothesis of autoregulation of coronary blood flow.

during exercise when heart work and cardiac oxygen requirements are increased, coronary blood flow also increases (17). Mechanisms to explain such regulation of coronary perfusion have been sought for some time. Although nervous control may account, in part, for such regulation, local control has been demonstrated in hearts with no neural or humoral connections (7, 24). Berne (6) has suggested that autoregulation of coronary blood flow may be mediated by adenosine. This hypothesis was based on the facts that adenosine is a potent coronary vasodilator (11); it crosses cellular membranes readily (20); it is produced in preference to 5'-IMP in the heart (15); it is deaminated rapidly in the coronary circulation to inosine (3), which has no dilatory action; and its degradation products are found in the perfusate of dinitrophenol-treated and hypoxic dog hearts (19). Berne (6) has postulated that in response to hypoxia decreased oxygen tension of the myocardial cell could lead to the breakdown of adenine nucleotides to produce adenosine. The nucleoside would diffuse through the interstitial fluid to resistance vessels to induce coronary dilation with resultant increased blood and oxygen supply to the myocardium; this would act as a feedback mechanism to reduce the formation of adenosine as oxygen tension returned to an adequate level. Although it has not been established unequivocally that adenosine regulates the regulation of coronary flow (1, 22), the formation of the nucleoside by the mammalian heart is well documented (15, 19, 26, 27). The nucleoside is produced by dephosphorylation of 5'-AMP by 5'-nucleotidase according to the reaction:

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

An understanding of the properties of this enzyme and of cardiac adenylate metabolism in general may yield meaningful information regarding the physiological significance of adenosine as a coronary dilator. Previously (4), we reported that 5'-nucleotidase activity was present in the hearts of a variety of mammalian species, and, in accord with the adenosine hypothesis, it was largely associated with cellular membrane structures. The present communication is concerned with a study of 5'-nucleotidase levels in hearts of several species; activities of two other enzymes which utilize 5'-AMP as substrate, adenylate deaminase and adenylosuccinate lyase, are also reported. In addition, several properties of 5'-nucleotidase solubilized from rat heart are examined, particularly those properties that may be relevant to physiological regulation of adenosine formation. Histochemical studies designed to examine the cellular localization of the enzyme are also presented.

MATERIALS AND METHODS

Adenosine deaminase (EC 3.5.4.4) (calf intestinal mucosa) was purchased from Calbiochem, Los Angeles. Swiss albino mice, Wistar rats, albino rabbits, and mongrel dogs were obtained from the University of British Columbia animal unit. Turtles were purchased from College Biological Supplies, Seattle; pigeons were obtained locally. Human papillary muscle was obtained as biopsy material through the courtesy of Dr. Peter Allen, Vancouver General Hospital.
Preparation of Extracts and Enzyme Assays

Hearts were removed from animals immediately after sacrifice, washed with 0.9% NaCl to remove blood, and used immediately or stored at -80 C.

5'-Nucleotidase. Fresh or frozen ventricle was minced with scissors and homogenized at 4 C in a Potter-Elvehjem homogenizer in 10 volumes of 50 mM 2-amino-2-methyl-1,3-propanediol, pH 9.0, containing 2.8 mM MgCl₂ and 0.15 mM KCl. The homogenate was strained through cheesecloth to remove connective tissue. To remove endogenous orthophosphate, 1 volume of saturated ammonium sulfate solution was added slowly to the homogenate with constant stirring. The mixture was centrifuged at 30,000 X g for 20 min, the supernatant fluid was discarded, and the walls of the centrifuge tube were carefully rinsed with buffer. The pellet was dispersed in a volume of the above buffer equivalent to that of the original homogenate. For assay, 20 μl of 0.10 mM 5'-AMP was added to 230 μl of the tissue suspension and the tubes were incubated for 10 min at 37 C with mechanical shaking. Control tubes were in which 5'-AMP was replaced with 3'-AMP, β-glycerophosphate, or water. All assays were performed in duplicate. The reaction was stopped by the addition of 1.0 ml of cold 3% trichloroacetic acid; after centrifugation to remove denatured protein, aliquots of the supernatant fluid were assayed for inorganic phosphate by the method of Fiske and SubbaRow (14). Under these conditions (pH 9.0), prior deamination of 5'-AMP by adenylic deaminase was not a complicating factor. In control experiments to examine this possibility, incubation mixtures were subjected to paper chromatography. Neither 5'-IMP nor inosine were present.

Adenylate deaminase. Fresh or frozen ventricular muscle was homogenized in 10 volumes of 0.1 mM potassium phosphate buffer, pH 7.3, containing 0.15 mM KCl, using a Potter-Elvehjem homogenizer. After centrifugation at 30,000 X g for 20 min, the clear supernatant was removed for assay. The incubation mixture contained 90 μl of 0.1 mM 5'-AMP, an appropriate amount of enzyme, and buffer (0.1 mM potassium phosphate, pH 7.5, containing 0.15 mM KCl). The final volume was 250 μl. After incubation for 10 min at 37 C, the reaction was stopped by the addition of 250 μl of 10% perchloric acid. A control tube was prepared in a manner identical to the experimental, except that the perchloric acid was added before the tissue extract. After the removal of denatured protein by centrifugation, 20-μl aliquots of each supernatant were diluted with water to a final volume of 1.0 ml and the absorbance at 265 nm was measured in a DU spectrophotometer (light path 1 cm), essentially as described by Nikiforuk and Colowick (25). The decrease in absorbance of the experimental tubes compared with the control was used to calculate the amount of substrate deaminated. The basis for the assay results from the fact that the molar extinction coefficient of 5'-IMP (the deamination product) is only 40% of that of 5'-AMP at this wave length. Activity was calculated as micromoles of substrate deaminated per minute per milligram of protein or per gram of tissue wet weight.

Adenylate kinase. Extracts of ventricle were prepared as for adenylate deaminase determinations except that the homogenizing buffer was 0.1 mM Tris-HCl, pH 7.0, containing 10 mM MgCl₂ and 0.1 mM KCl. For activity determinations, ADP was used as substrate and the 5'-AMP formed was deaminated by coupling with excess purified adenylic deaminase (21). Thus, to spectrophotometric cells (d = 0.5 cm) was added 50 μl of 0.01 mM ADP, 20 μl of adenylic deaminase (75 μg protein, sufficient to deaminate 1.5 μmoles 5'-AMP/min under these conditions), and buffer (0.1 mM Tris-HCl, pH 7.0, containing 10 mM MgCl₂ and 0.10 mM KCl) to a final volume of 1.5 ml. The temperature was 37 C. A blank contained all components of the assay except substrate. The reaction was started by addition of suitably diluted heart extract and the rate of decrease in absorbance at 265 nm was determined using a Unicam SP 800 recording spectrophotometer with a 1.0 A attenuator (to allow the use of high substrate concentrations). Activity was calculated as micromoles of ADP utilized per minute per milligram of protein or per gram of tissue wet weight.

Each of the above enzymes were assayed under conditions of substrate saturation and, where necessary, optimal metal ion concentrations. Except for 5'-nucleotidase, the pH value chosen for each assay was near physiological pH.

Assay of Partially Purified 5'-Nucleotidase

For the studies on partially purified 5'-nucleotidase, a wide range of conditions and substrate concentrations were required. Two different assays were used to accommodate all of the experimental conditions. Thus with substrate (5'-AMP) concentrations of 0.16 mM and higher, activity was determined by an assay similar to that previously described for crude extracts (determination of liberated inorganic phosphate, except that the buffer was 50 mM Tris HCl, pH 7.2). It was not possible to employ substrate concentrations lower than 0.16 mM because of limitations in the sensitivity of the phosphate determination. For 5'-AMP concentrations less than 0.16 mM, enzymic activity was determined by a more sensitive, direct optical assay that we have described previously (4). The method depends on the deamination of the product adenosine, by coupling the reaction with excess purified adenosine deaminase and measurement of the resulting decrease in absorbance at 265 nm. Incubations were carried out in spectrophotometric cells at 37 C; the rate of decrease in absorbance at 265 nm was followed in a Unicam SP 800 spectrophotometer with a slave recorder attachment. This assay was limited by the low signal-to-noise ratio at high substrate concentrations.

Protein determinations were performed by the biuret method (see ref. 23), except in the case of partially purified enzyme preparations for which the optical method of Warburg and Christian (see ref. 23) was used.

Partial Purification of 5'-Nucleotidase

The enzyme was extracted from an acetone powder of rat hearts prepared as previously described (4). Acetone powder (1.5 g) was mixed with 10 ml of 50 mM Tris-HCl, pH 7.5 at 4 C, in a glass mortar until a smooth paste was obtained. The paste was transferred to a Potter-Elvehjem homogenizer, 40 ml of buffer was added and the mixture was homogenized thoroughly. The suspension was centrifuged at 30,000 X g for 10 min and the supernatant fluid was discarded. The pellet was reextracted with an additional 50 ml of the above buffer and, after centrifugation as
before, the supernatant fluid was discarded. The pellet was then extracted 9 times with 50 ml of 50 mm Tris-HCl, pH 7.5, containing 2 mm KBr, each clear supernatant being discarded after centrifugation at 30,000 X g. In order to remove remaining KBr, the pellet was extracted two more times with 50 ml of 50 mm Tris HCl, pH 7.5, containing 1% sodium deoxycholate. The suspension was stirred for 3 hr at 4 C and centrifuged at 30,000 X g for 10 min. The supernatant that contained the enzyme was set aside at 4 C. The pellet was extracted once more with 3 ml of 50 mm Tris-HCl, pH 7.5, and after centrifugation the two supernatants were combined. This extract was brought to 15% saturation with respect to ammonium sulfate by the addition of an appropriate volume of a saturated solution of this salt. The mixture was centrifuged at 30,000 X g for 10 min and the clear supernatant was dialyzed against 30 volumes of 50 mm Tris-HCl, pH 7.5, for 3 hr with two changes of buffer. For experiments in which it was necessary to remove endogenous divalent cation the solution was brought to 10 mm EDTA prior to dialysis.

Histochemistry

5'-Nucleotidase was identified by the method of Wachstein and Meisel (31), which depends on fixation of liberated inorganic phosphate as the lead salt. Fresh tissue was frozen and cut into sections 10-μ thick which were mounted on glass coverslips. The preparations were incubated for 45 min at 37 C in a medium containing 4 ml of 50 mm Tris maleate, pH 7.0, 1 ml of 0.1 M MgSO4, 0.6 ml of 9% lead nitrate, 0.4 ml of water, and 4 ml of 2.5 mm 5'-AMP as the neutralized potassium salt. Control experiments contained 4 ml of 3'-AMP (2.5 mm), β-glycerol phosphate (4 mm), or water. The reactions were stopped by placing the sections in 10% Formal saline (4% formaldehyde in 0.9% saline). After washing with water the preparations were immersed in dilute ammonium sulfide (31), washed thoroughly with water, mounted on microscope slides with glycerine jelly, and sealed with nail lacquer.

RESULTS

If adenosine is the mediator of autoregulation it might be expected that animals that require large changes in coronary flow would be able to produce proportionately large amounts of the nucleoside. Thus, animals that effect large and rapid changes in heart load, for example birds, might have higher levels of 5'-nucleotidase than animals such as reptiles that maintain rather constant loads on their hearts. We know of no studies relating coronary flow to rate of oxygen consumption in hearts of these species, however. 5'-Nucleotidase activity in hearts of several species is shown in Fig. 1. Large differences in enzyme levels were observed among the species examined and were evident whether activity was expressed on the basis of protein or tissue wet weight. Rat heart possessed the highest activity, followed by the dog, mouse, and rabbit. Homogenates of turtle ventricle contained no detectable 5'-nucleotidase. However, a nonspecific phosphatase was present in this tissue as evidenced by phosphate liberation equivalent to 5.4 μmoles/70 min per mg protein when either 3'-AMP or β-glycerol phosphate was used in the assay. Under the conditions used here, pigeon ventricle homogenates contained no detectable 5'-nucleotidase. These data would seem to indicate that adenosine forming activity is not related to capacity to effect changes in myocardial flow.

In addition to 5'-nucleotidase, certain other enzymes of 5'-AMP metabolism could affect adenosine formation in the cell. Two of these, adenylyl kinase and adenylyl deaminase, are most relevant; the former catalyzes adenine nucleotide interconversion and the latter converts 5'-AMP to 5'-IMP. Adenylyl deaminase levels in heart homogenates of various species is shown in Fig. 2. This enzyme was present in all cases except dog hearts. Turtle ventricle contained at least 5 times as much activity as that of the rat, rabbit, or pigeon. Activity in all hearts was much lower than that present in rabbit skeletal muscle, which is presented for comparative purposes. Adenylyl kinase activity in ventricular tissue is presented in Fig. 3. Pigeon ventricle possessed the highest activity of this enzyme followed by rabbit, dog, rat, and turtle. Again, activity levels in all hearts were significantly lower than in rabbit skeletal muscle. The fact that turtle heart possessed low adenylyl kinase and high adenylyl deaminase may suggest that the heart of this species has a relatively more anaerobic metabolism than mammalian hearts. Pigeon heart possessed high levels of adenylyl kinase.

Histochemical Localization of 5'-Nucleotidase

Rubio and Berne (29) have suggested that myocardial cells release adenosine continuously into the surrounding
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interstitial fluid. Rostgaard and Behnke (28) have examined tissue sections of prefixed rat hearts by electron microscopy and demonstrated the presence of phosphate liberation from 5'-AMP in intercellular spaces and the T-system complex. We have examined the localization of the enzyme in hearts of various species using unfixed sections processed by the procedure of Wachstein and Meisel (31). Using this method, considerable lead deposition (which delineates the site of phosphate release) was observed when mammalian ventricular sections were incubated with 5'-AMP. The enzyme activity was discretely localized in specific regions throughout the entire ventricle. The uniformity of this distribution is illustrated in Fig. 4A, a low-power magnification photomicrograph of rat ventricle section incubated with 5'-AMP. Examination at higher magnification (Fig. 4B) revealed that lead deposition occurred predominantly in structures that surround muscle cells. That these deposits reflected the activity of a specific 5'-nucleotidase and not a nonspecific phosphatase is apparent on inspection of Fig.

**FIG. 2.** Adenylate deaminase in hearts of several species and rabbit skeletal muscle. Activity is expressed as micromoles 5'-AMP deaminated per minute per milligram protein (open bars) and per gram of tissue wet weight (hatched bars).

**FIG. 3.** Adenylate kinase activity in hearts of various species and rabbit skeletal muscle. Activity is presented on basis of protein (open bars) and on tissue wet weight (hatched bars). Vertical lines represent ± SEM.

**FIG. 4.** Histochemical localization of 5'-nucleotidase in ventricle sections. A: rat ventricle, substrate 5'-AMP, magnification ×50. B: rat ventricle, substrate 5'-AMP, magnification ×530. C: rat ventricle, control (3'-AMP was substituted for 5'-AMP), magnification ×530. D: human papillary muscle, substrate 5'-AMP, magnification ×530. Note longitudinally sectioned endothelial cells with high 5'-nucleotidase activity. E: human papillary muscle, control (3'-AMP substituted for 5'-AMP), magnification ×530. Identical results were obtained with sections incubated with β-glycerol phosphate or water. F: mouse ventricle, substrate 5'-AMP, magnification ×530. G: guinea pig heart, stained with hematoxylin and eosin, magnification ×290. H: guinea pig ventricle, substrate 5'-AMP, magnification ×50.
4C in which 3'-AMP replaced 5'-AMP during incubation (compare with Fig. 4B). 5'-Nucleotidase of human papillary muscle was also found primarily in structures between muscle cells (Fig. 4D). This activity, too, seemed due to a specific 5'-nucleotidase because the control in which 3'-AMP was used (Fig. 4E) contained no lead deposits. In sections of mouse ventricle (Fig. 4F), the enzyme was also found between muscle cells. Figure 4G, a hematoxylin- and cosin-stained section of guinea pig heart, revealed that those structures demonstrating 5'-nucleotidase activity contained nuclei and we conclude that they are endothelial cells of capillaries. In some instances cell membranes of cardiac cells are clearly visible (Fig. 4B, D, and F) and are essentially devoid of lead deposition. Thus it seems that much of the 5'-nucleotidase of mammalian ventricles appears to be localized in the capillary endothelium. It should be emphasized that we have used unfixed tissue preparations. It is conceivable that 5'-nucleotidase residing in sarcolemmal sites may be more discretely identified by methods that employ fixatives. Very recently, Borgers, Schaper, and Schaper (9) have reported that no 5'-nucleotidase was present within the myocardial cells, but the interstitial cells reacted intensely to histochemical staining. In guinea pig ventricle, 5'-nucleotidase was also found in walls of larger blood vessels as well as in capillaries (Fig. 4H). No lead deposits were observed when pigeon ventricle sections were incubated with 5'-AMP using the present method. Turtle ventricle sections showed evidence of nonspecific phosphatase activity; faint lead deposition occurred whether 5'-AMP, 3'-AMP, or P-glycerol phosphate was used, and the activity was not confined to endothelial cells.

Properties of Cardiac 5'-Nucleotidase

5'-Nucleotidase is a ubiquitous enzyme and has been studied in a variety of cells and tissues (10). Our earlier studies (4) revealed that most of the activity in rat heart was membrane bound and could be solubilized with deoxycholate. ATP was found to be a powerful inhibitor of the enzyme. We have examined several properties that may have relevance in regulation of adenosine formation during hypoxia. The enzyme was extracted from acetone powders prepared from rat hearts (see MATERIALS AND METHODS). Soluble protein, including soluble 5'-nucleotidase, was extracted from membrane-bound activity. After the removal of additional nonenzyme protein with 2 m KBr, the particulate enzyme was solubilized with deoxycholate. The final preparation hydrolyzed 5'-AMP at a rate of 0.15 μmoles/min per mg of protein and represented a purification of about 20 fold over the acetone powder with an apparent yield of 107%. This high yield is likely due in part to the solubilizing action of deoxycholate and may represent enhanced availability of the active site for substrate. Although the purification was modest, the preparation was sufficiently free of ATPase, nonspecific phosphatase, adenylate deaminase, adenylate kinase, and adenosine deaminase so that interference with the 5'-nucleotidase assay was obviated. Further efforts to purify the enzyme based on chromatography on DEAE-cellulose, CM-cellulose, gel filtration on Sepharose 6B, solvent fractionation, and isoelectric precipitation were unsuccessful.

Effect of Divalent Cations

To ensure absence of endogenous metal ions, enzyme preparations were treated with 10 nm EDTA before dialysis. Such preparations possessed significant 5'-nucleotidase activity in the absence of added divalent cation. Activity was greatly increased by the addition of Mg2+, Ni2+, or Mn2+ (Fig. 5). Enzyme activity was maximal at concentrations of Mg2+, Mn2+, and Ni2+ of 16, 2, and 1 mm, respectively. Mn2+ caused greater stimulation of enzyme than Mg2+ and was inhibitory at concentrations above 2 mm. The KA for Mg2+ obtained from a Hofstee plot (velocity vs. (velocity/Mg2+ concentration)) of the data in Fig. 5 was 1.9 mm. Ca2+ (10 mm) had only a slight stimulatory effect and in the presence of Mg2+ it did not alter enzyme activity. These results are markedly different from those of Edwards and Maguire (12), who have reported that both Mg2+ and Ca2+ inhibited enzyme activity; half maximal inhibition was obtained at Ca2+ and Mg2+ concentrations of 3 and 8 mm, respectively. Previously (4) we found that Ca2+ (7 mm) was inhibitory when the assay was conducted at pH 8.7 in the absence of Mg2+.

Substrate Specificity

Rat ventricular 5'-nucleotidase possessed a broad substrate specificity for nucleoside 5'-monophosphates (Fig. 6). This is typical of the enzyme from most sources (10). 5'-AMP was not the preferred substrate, 5'UMP was hydrolyzed more rapidly. The 3'-deoxyribo nucleoside 5'-monophosphates were hydrolyzed at about half the rate...
of the corresponding ribonucleoside 5'-monophosphates. In every case, enzyme activity was significantly increased by Mg²⁺ (16 mM) (Fig. 6). Phosphate esters not bearing base moieties were not attacked by the semipurified enzyme. Thus, ribose-5-phosphate, glucose-6-phosphate, fructose-1-phosphate, fructose-1,6-diphosphate, ribulose-5-phosphate, and galactose-6-phosphate did not serve as substrates. In addition, neither p-nitrophenylphosphate nor pyrophosphate was attacked. The broad substrate specificity did not appear to be due to the presence of more than one enzyme. Thus the relative rates of hydrolysis of substrates were similar in the presence and absence of Mg²⁺ (Fig. 6). Furthermore, 5'-UMP, a pyrimidine nucleotide, competitively inhibited the dephosphorylation of 5'-AMP, indicating the interaction at the same catalytic site.

**Affinity for Substrate**

Previously (4) we determined the \( K_m \) of the heart enzyme for 5'-AMP to be 18 \( \mu \)M. In the present experiments, the effect of substrate was examined over the range 3.3–10 mM in the presence and absence of 16 mM MgCl₂ using the optical assay for velocity measurements with substrate concentrations less than 0.16 mM and the assay based on phosphate liberation for substrate concentrations above this (see Methods and Materials). The \( K_m \) was calculated from double-reciprocal plots to be 21 \( \mu \)M both in the presence and absence of Mg²⁺. Thus, stimulatory action of Mg²⁺ on enzyme activity was due to an increase in \( V_{max} \), there being no change in affinity for substrate. The \( K_m \) value of 21 \( \mu \)M is in good agreement with our previous value of 18 \( \mu \)M determined in the presence of Mg²⁺ and also with that determined by Edwards and Maguire (14.5 \( \mu \)M) (12) and by Sullivan and Alpers (23 \( \mu \)M) (30), who conducted their experiments in the absence of Mg²⁺.

**Effect of Nucleoside Di- and Triphosphates**

Previously (4), we showed that cardiac 5'-nucleotidase was markedly inhibited by ATP and suggested that this may have significance in regulating adenosine formation. In the anoxic heart, when ATP levels fall, adenosine formation might increase as a result of removal of inhibitory constraint. More recently, however, it has been shown that ADP is actually a more potent inhibitor than ATP (30). In the presence of 16 mM MgCl₂, 5'-nucleotidase was inhibited by both ADP and ATP (Fig. 7). It was unlikely that inhibition was due to chelation of Mg²⁺, because the cation was present at concentrations 16 and 32 times that of ATP or ADP, respectively. ADP was the more effective inhibitor; at 2 mM 5'-AMP, inhibition by 0.5 mM ADP was 41%, whereas that produced by 1.0 mM ATP was 11%. ADP inhibition was also examined when the assay was performed in the absence of Mg²++. Inhibition in the absence of Mg²⁺ appeared to be of the mixed (competitive-noncompetitive) type as revealed by the Hofstee plot (18) (velocity vs. (velocity/substrate concentration)) (Fig. 8). The maximum velocity was decreased by 25% at an ADP concentration of 3.3 \( \mu \)M while the apparent \( K_m \) increased from 21 to 30 \( \mu \)M. These data are in agreement with the results of Edwards and Maguire (12), who found that nucleoside triphosphates inhibited the cardiac enzyme by altering both the maximum velocity and the \( K_m \) when assays were performed in the absence of Mg²⁺. In contrast, Sullivan and Alpers (30) reported that both ADP and ATP inhibited the enzyme in a manner competitive for substrate; their assays were also performed in the absence of Mg²⁺. When assays were performed in the presence of Mg²⁺ (16 mM), ADP produced inhibition in a noncompetitive manner (Fig. 9). The maximal velocity was decreased 45% by 33 \( \mu \)M ADP while the affinity for 5'-AMP was not affected. The calculated value

![Fig. 6. Substrate specificity of rat heart 5'-nucleotidase. Open bars represent orthophosphate liberated in presence of 16 mM MgCl₂; hatched bars in absence of added divalent cation. All values are expressed as percent of maximum activity, 1.0 ml.](http://ajplegacy.physiology.org/doi/10.1152/ajplegacy.1978.42.3.11)

![Fig. 7. Inhibition of 5'-nucleotidase by ATP and ADP. Activity was measured by liberation of orthophosphate. Assay contained MgCl₂ 16 mM, enzyme 19 \( \mu \)g protein, and 5'-AMP varied as indicated. ○, control; △, ATP present at 1 mM; □, ADP present at 0.5 mM.](http://ajplegacy.physiology.org/doi/10.1152/ajplegacy.1978.42.3.11)
of the $K_i$ for ADP was 40 $\mu M$. These results differ from those of Sullivan and Alpers (30), who reported that ADP at 1.7 $\mu M$ produced no inhibition in the presence of 8 mM MgCl$_2$. ATP in the presence of 16 mM MgCl$_2$ did not inhibit to the same extent as ADP. When the substrate concentration was 33 $\mu M$, ATP at 33 $\mu M$ inhibited activity 11%, whereas ADP at the same concentration produced 35% inhibition. Rat ventricle 5'-nucleotidase was inhibited by orthophosphate in a noncompetitive manner, whereas 5'-UMP inhibited in a manner competitive with substrate (Fig. 10). The $K_i$ value for orthophosphate was calculated to be 73 mM.

**DISCUSSION**

The data from both the direct assays and histochemistry reveal an extremely wide range of 5'-nucleotidase activity in hearts of various species, rat heart being the most active while turtle and pigeon hearts seemed devoid of activity. This might indicate that adenosine is not universally involved in mediating coronary autoregulation. The coronary dilatory action of adenosine is readily demonstrated in mammalian hearts using the Langendorff perfusion technique. In separate experiments we found that adenosine at concentrations as high as 10 mM in the perfusion fluid did not increase blood flow in turtle hearts. The relatively high level of adenylate deaminase in turtle ventricle would indicate that in this tissue 5'-AMP is catabolized by a route more analogous to that in skeletal muscle (19). This may be indicative of a more anaerobic energy metabolism in the heart of this species (5). Pigeon ventricle did not hydrolyze 5'-AMP by the methods used here. This tissue, however, possesses 5'-nucleotidase (16), but the enzyme is located exclusively in the soluble cytoplasm. In view of the high
level of adenyate kinase in pigeon ventricle, it would seem that this tissue may be adapted to conserve adenine nucleotides in the form of high-energy phosphates. This would be consistent with a highly developed capacity for acrobic metabolism and function.

The finding that intense 5′-nucleotidase activity was associated with capillary endothelial cells and with the walls of larger blood vessels in mammalian hearts would have considerable relevance with respect to the autoregulatory role of adenosine. Previously Essner, Novikoff, and Quintana (13) have suggested that a nonspecific phosphatase active at pH 5.0 could be identified in the microvilli of capillary endothelial cells. It is likely that this activity is different from the specific 5′-nucleotidase described here because in their experiments staining was also obtained with β-glycerolphosphate as substrate. Using electron microscopic examination of fixed preparations, Rostgaard and Behnke (28) have shown that 5′-nucleotidase activity is present within the cardiac cell being located in the T-system complex. Their electron photomicrographs show much more intense lead deposition in regions described as intercellular spaces. The cellular localization described here could explain observations previously made in this laboratory that 5′-AMP was dephosphorylated by a single passage through the coronary circulation of the perfused rat heart (3). It was suggested at that time that enzymes that convert adenosine nucleotides to adenosine must exist within or on the coronary vascular cells. Our results raise the possibility that the source of adenosine for the regulation of coronary flow may be endothelial cells of capillaries rather than muscle cells. Perhaps reduced PO2 of the capillaries rather than of the cardiac cell per se may be the signal for adenosine formation. In terms of increased work, muscle cells, by greater oxygen utilization, could increase the PO2 gradient from capillaries causing the latter to form adenosine, which would then diffuse to the region of the precapillary resistance vessels and induce vasodilation. This indirect signal for adenosine formation may be advantageous in that it would tend to conserve adenosine within the cardiac cell where it is needed for muscular work and other energy-requiring processes. Rubio and Berne (29) have suggested that adenosine is restricted to the interstitial spaces, the implication being that it is delivered there after formation by 5′-nucleotidase existing in the cardiac cell membrane. Whatever the site of formation, the crucial question is: does the diffusion of adenosine into the interstitial fluid occur at a rate sufficient to cause dilution of the appropriate precapillary resistance vessels. The possibility of adequate delivery of adenosine to this critical site must surely be as likely by 5′-nucleotidase located in endothelial cells as in plasma membrane of cardiac cells. It should be mentioned that these histochemical studies do not exclude the possibility that 5′-AMP could first be deaminated to 5′-IMP prior to dephosphorylation. The latter nucleotide is a substrate for 5′-nucleotidase.

Considerable caution is needed in interpreting the in vivo significance of enzymatic regulatory mechanisms uncovered from in vitro studies. In the present experiments, the tissue was subjected to acetone extraction and the enzyme was subsequently solubilized by detergent; both are relatively harsh treatments. Furthermore it is likely impossible to reproduce in vitro the precise in vivo environment, especially of a membrane-bound enzyme. With these reservations, certain properties of the rat heart 5′-nucleotidase as revealed by studies of the semipurified enzyme seem to suggest that enzyme activity and adenosine formation may be enhanced during cardiac hypoxia and diminished during adequate oxygenation. One characteristic which may be interpreted as support for the adenosine hypothesis is the positive correlation between Mg2+ concentration and enzyme activity. Since ATP is an excellent chelator of Mg2+ (32), the cellular concentration of this tripositive could determine the availability of this divalent cation for binding to the enzyme. It might be expected that when tissue oxygenation is adequate and the energy charge of the adenylate pool is high, enzyme activity could be constrained by virtue of Mg2+-binding by high intracellular ATP. Conversely, at lowered energy charge, Mg2+ could become available for binding to the enzyme with consequent increased adenosine formation. 5′-Nucleotidase is strongly inhibited by ATP and we have previously suggested (4) that this may constitute a physiological mechanism for regulation of adenosine formation. Thus 5′-nucleotidase activity could be suppressed by high-energy charge when tissue oxygenation is adequate and deinhibited during periods of anoxia when energy charge of the adenylate pool is reduced. However, this possibility is now complicated by the fact that ADP inhibits the enzyme and, in fact, is a more powerful inhibitor than ATP. The immediate product of ATP hydrolysis is ADP and the possibility therefore exists that adenosine formation could actually be inhibited by lowered energy charge. In addition, orthophosphate, which is also a product of adenine nucleotide hydrolysis, inhibits the enzyme. Thus ADP and orthophosphate, which both increase during hypoxia (39), could act to reduce adenosine production in the hypoxic myocardium. These observations, of course, cast doubt on our proposed regulatory role of ATP (4) and may even be considered to cast doubt on the validity of the adenosine hypothesis. It must be remembered, though, that in hypoxia 5′-AMP levels increase, that is, ADP is further suppressed by high-energy charge when tissue oxygenation is adequate and the energy charge of the adenylate pool (2) is high, enzyme activity could be constrained by virtue of Mg2+-binding by high intracellular ATP. The immediate product of ATP hydrolysis is ADP and the possibility therefore exists that adenosine formation could actually be inhibited by lowered energy charge. Perhaps reduced PO2 of the capillaries rather than of the cardiac cell per se may be the signal for adenosine formation. In terms of increased work, muscle cells, by greater oxygen utilization, could increase the PO2 gradient from capillaries causing the latter to form adenosine, which would then diffuse to the region of the precapillary resistance vessels and induce vasodilation. This indirect signal for adenosine formation may be advantageous in that it would tend to conserve adenosine within the cardiac cell where it is needed for muscular work and other energy-requiring processes. Rubio and Berne (29) have suggested that adenosine is restricted to the interstitial spaces, the implication being that it is delivered there after formation by 5′-nucleotidase existing in the cardiac cell membrane. Whatever the site of formation, the crucial question is: does the diffusion of adenosine into the interstitial fluid occur at a rate sufficient to cause dilution of the appropriate precapillary resistance vessels. The possibility of adequate delivery of adenosine to this critical site must surely be as likely by 5′-nucleotidase located in endothelial cells as in plasma membrane of cardiac cells. It should be mentioned that these histochemical studies do not exclude the possibility that 5′-AMP could first be deaminated to 5′-IMP prior to dephosphorylation. The latter nucleotide is a substrate for 5′-nucleotidase.

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