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Purification and Properties of Rat Heart 5′-Nucleotidase

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SUMMARY

5′-Nucleotidase (EC 3.1.3.5) has been purified 26-fold from rat heart and obtained substantially free of nonspecific phosphatase activity. The pH optimum for the enzyme was 7.6; at this pH, 7 mM Mg^{++} and 2 mM Ca^{++} caused 40% inhibition of 5′-nucleotidase activity, and 0.17–0.69 mM Mg^{++} was without effect on activity. The nucleoside triphosphates ATP, UTP, CTP, GTP, and ITP inhibited 5′-nucleotidase in a noncompetitive manner with respect to adenosine 5′-monophosphate. The AMP analogues adenosine 5′-phosphorothioate (AMPS) and 2-chloroadenosine 5′-monophosphate (2-chloro-AMP), which are vasodilators, and 2-methylthioadenosine 5′-monophosphate (2-methylthio-AMP), which has no vasodilatory properties, were all substrates of 5′-nucleotidase, with Michaelis constants similar to that of AMP. The maximum velocities of hydrolysis of AMPS, 2-chloro-AMP, and 2-methylthio-AMP were 36%, 79%, and 116%, respectively, of the maximum velocity for AMP. The rates of hydrolysis of these analogues by 5′-nucleotidase are considered in relation to their vasodilatory effects.

INTRODUCTION

Adenosine 5′-monophosphate and its analogues 2-chloroadenosine 5′-monophosphate and adenosine 5′-phosphorothioate are vasodilators (1),[3] while the related analogue 2-methylthioadenosine 5′-monophosphate has no discernible vasodilatory properties (2). The ratio of potencies of AMP, AMPS,[4] and 2-chloro-AMP as vasodepressors in the rat is 1:0.8[3]:7, respectively (1). The vasodilatory effect of AMP is brief, that of AMPS lasts longer, and the depression of blood pressure caused by 2-chloro-AMP is of even greater duration. The rates of hydrolysis *in vivo* of AMP, AMPS, 2-chloro-AMP, and 2-methylthio-AMP to their parent nucleosides, adenosine, 2-chloroadenosine, and 2-methylthioadenosine, which all have vasodilator properties (1, 2), may be pertinent to both the vasodilatory potency and duration of action of these nucleotides.

This paper reports the 26-fold purification from rat heart of 5′-nucleotidase (EC 3.1.3.5) substantially free from nonspecific phosphatase activity, studies of the substrate specificity of the enzyme for the vasodilatory nucleotide analogues, and studies of the general properties of the enzyme, particularly the nature of the inhibition by the nucleoside triphosphates ATP, GTP, ITP, UTP, and CTP.

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[3] F. Michal and F. Penglis, unpublished observations.

[4] The abbreviations used are: AMPS, adenosine 5′-phosphorothioate; 2-chloro-AMP and 2-methylthio-AMP, the 5′-monophosphates of 2-chloroadenosine and 2-methylthioadenosine.

5′-Nucleotidase has been partially purified from a number of mammalian sources, including human and rat liver (3, 4), bovine pituitary gland (5), calf intestinal mucosa (6), and sheep brain (7), and a 12-fold purification of the enzyme from rat heart acetone powder has been reported (8).

MATERIALS AND METHODS

Materials

2-Chloro-AMP (1) and 2-methylthio-AMP (2) were synthesized in this institute, and AMPS (9) was a gift from Professor M. R. Atkinson. AMP, GMP, and nucleoside triphosphates were Sigma products, and IMP was obtained from Pierce Biochemicals. Calf intestinal adenosine deaminase was obtained from Sigma Chemical Company; the specific activity at pH 7.6 and 30° was 270 units (micromoles of adenosine deaminated per minute) per milligram of protein. Whatman cellulose phosphate and DEAE-cellulose DE 22 were used and were cycled before use according to instructions given by the manufacturer. Sephadex G-200 was obtained from Pharmacia. All reagents and buffers were analytical grade.

Enzyme Assays

1. When nonspecific phosphatase was present, the assay mixture contained 2 μmoles of substrate, 30 μmoles of Tris-HCl (pH 7.6), and enzyme in a total volume of 1.2 ml. After 10 min of incubation at 30°, the reaction was stopped by the addition of 1 ml of 10% trichloracetic acid. Precipitated protein was removed by centrifugation, and 1 ml of the supernatant fluid was used to estimate inorganic phosphate as described by Martin and Doty (10). Phenyl phosphate was the substrate for nonspecific phosphatase, and the phosphate released by 5′-nucleotidase was obtained from the difference between total phosphate liberated from AMP and that from phenyl phosphate. Blank values were obtained by adding trichloracetic acid prior to the enzyme. Assays were carried out in duplicate or triplicate. In kinetic studies with the purified enzyme, the reaction was stopped by the direct addition of the acid ammonium molybdate reagent to the assay mixture.

2. When the enzyme preparation was free from detectable nonspecific phosphatase, 5′-nucleotidase was measured by coupling the reaction with excess adenosine deaminase and following the change in optical density resulting from the conversion of adenosine to inosine (11). The assay mixture in a 1-cm light-path cuvette contained the enzyme (0.05–0.5 ml in 0.01 M Tris-HCl, pH 7.6), 0.24 μmole of AMP, 0.27 unit of adenosine deaminase, and 129 μmoles of Tris-HCl buffer, pH 7.6, in a final volume of 2.9 ml. The reaction was initiated by the addition of 5′-nucleotidase, and the absorbance change at 265 mμ was measured using a Gilford model 220 absorbance indicator attached to a Unicam monochromator and a Varian G2000 recorder. The cuvette compartment was thermostated at 30°. The absorbance change per minute was converted to micromoles of AMP hydrolyzed per minute by the following relationship:

$$\mu\text{moles/min} = \frac{\Delta A \times 2.9}{8.1}$$

where 2.9 is the volume of the assay mixture and 8.1 is derived from the difference in the extinction coefficients of adenosine and inosine at 265 mμ (11). Assays for kinetic studies were carried out in triplicate.

Unit of Activity

One unit of 5′-nucleotidase activity was defined as the amount of enzyme needed to dephosphorylate 1 mμmole of AMP per minute under the above assay conditions. The colorimetric assay and the spectrophotometric assay gave similar results.

Protein Assay

Protein was determined colorimetrically by the method of Lowry *et al.* (12), using bovine serum albumin as standard. The relative protein concentration of column eluates was estimated by measuring the optical density at 280 mμ.

Purification of 5′-Nucleotidase

Rats were killed by a blow on the head, and the hearts were excised, minced finely with scissors, washed with cold distilled water, and frozen. All steps in the purifica-

tion procedure were carried out at 0–4°, and all buffers used contained 1 mM 2-mercaptoethanol.

Step I. Fourteen grams of frozen hearts were homogenized in 2 volumes of distilled water at speed I in a Sorvall Omnimixer for 45 sec. The homogenate was diluted to 100 ml with distilled water and centrifuged at 34,000 × *g* for 1 hr. The supernatant fluid was discarded, and the precipitate was resuspended in 50 ml of aqueous 3% sodium deoxycholate by gentle hand homogenization in a Potter-Elvehjem homogenizer. The suspension was stirred for 30 min and then centrifuged at 34,000 × *g* for 1 hr. The precipitate was discarded, and the supernatant fraction (60 ml) was used directly in step II.

Step II. Acetone at −30° to −40° was added dropwise to the stirred enzyme solution to give a final acetone concentration of 15% (v/v). Stirring was continued for 10 min; precipitated protein was allowed to flocculate for a further 10 min and was then removed by centrifuging at 10,000 × *g* and −6° for 20 min. The precipitate was discarded, and the supernatant fraction (60 ml) was dialyzed immediately against 0.01 M phosphate buffer, pH 6.6.

Step III. Cellulose phosphate (4.5 g) equilibrated in 30 ml of 0.01 M phosphate buffer, pH 6.6, was added to the enzyme solution. The suspension was stirred for 30 min and centrifuged at 10,000 × *g* for 10 min. The supernatant fluid was decanted, and the cellulose phosphate was resuspended in 30 ml of the same buffer. The suspension was stirred and centrifuged as before, and the supernatant fractions were combined and dialyzed against 0.01 M Tris-HCl buffer, pH 7.9.

Step IV. A slurry of DEAE-cellulose DE 22 (6.0 g) equilibrated in 0.01 M Tris-HCl buffer, pH 7.9, was added with stirring to the enzyme solution from step III until no more 5′-nucleotidase activity remained in solution. The DEAE-cellulose was then poured into a column, washed with the same buffer, and then eluted with 100 ml of buffer followed by 100 ml of buffer containing 0.1 M KCl and 300 ml of buffer containing 0.4 M KCl; 10-ml fractions were collected. The enzyme, associated with colored material, was eluted by 0.4 M KCl (Fig. 1). The active fractions were combined and dialyzed against 0.01 M β,β′-dimethyl glutarate buffer, pH 6.2.

Step V. The enzyme solution was applied to a column of 3.0 g of carboxymethylcellulose (1 g/20 mg of enzyme protein) which was equilibrated in the same buffer. The enzyme was eluted with buffer as a single protein peak; 10 ml fractions were collected, and active fractions were combined and dialyzed against 0.01 M Tris-HCl buffer, pH 7.6, for kinetic studies.

The purification procedure and elimination of nonspecific phosphatase activity is summarized in Table 1.

RESULTS

General Properties of Rat Heart 5′-Nucleotidase

The purified enzyme had a specific activity of 110 units/mg of protein, and was stable in 0.01 M Tris-HCl, pH 7.6, at 4° for 3 weeks. When the enzyme was filtered through a Sephadex G-200 column it was eluted in a

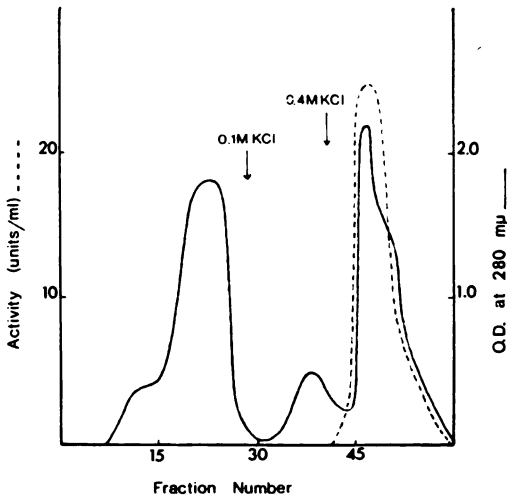

FIG. 1. *Elution pattern for 5′-nucleotidase from DEAE-cellulose*

The enzyme (156 mg of protein containing 5200 units), in 0.1 M Tris-HCl buffer, pH 7.9, was adsorbed to 6.0 g of DEAE-cellulose. Active protein was eluted with buffer containing 0.4 M KCl. Recovery of enzyme units was 76%. Enzyme activity was assayed by the spectrophotometric method as described in MATERIALS AND METHODS.

TABLE 1

Purification of 5′-nucleotidase from rat heart

Fraction	Volume	Protein	Total units[a]		Specific activity	Purification	Yield
			Nonspecific phosphatase	5′-Nucleotidase			
	ml	*mg*			*units/mg*	*-fold*	%
Homogenate	100	3,650	15,200	15,500	4.2		
Deoxycholate supernatant	60	1,480	6,500	11,800	8.0	1.9	76
Acetone supernatant	55	451	1,400	7,610	16.9	4.0	49
Cellulose phosphate supernatant	87	156	—[b]	5,200	33.3	7.9	34
DEAE-cellulose eluate	120	60	—[b]	3,950	65.8	15.5	26
CM-cellulose eluate	110	33	—[b]	3,630	110.0	26.2	24

[a] One unit is the amount of enzyme activity which hydrolyzes 1 mμmole of substrate per minute.

[b] No nonspecific phosphatase activity could be detected. The lower limit of the assay was 1 mμmole of phosphate formed per minute, so that the nonspecific phosphatase activity of these fractions was less than 5% of the 5′-nucleotidase activity.

sharp peak following the void volume, and no increase in specific activity was obtained.

Effect of Enzyme Concentration

With AMP as substrate in the spectrophotometric assay, the dependence of reaction rate on enzyme concentration was linear up to 340 μg of protein. In the colorimetric assay the maximum amount of purified 5′-nucleotidase used was 34 μg of protein, and with AMP, IMP, GMP, 2-chloro-AMP, 2-methylthio-AMP, and AMPS as substrates the relationship of rate to enzyme concentration was linear up to this limit.

Effect of pH and Metal Ions on Activity

5′-Nucleotidase exhibited a sharp optimum at pH 7.6 with AMP as substrate (Fig. 2). At this pH the presence of 170–690 μM Mg^{++} had no effect on enzyme activity, but at higher Mg^{++} levels inhibition which showed sigmoidal dependence on Mg^{++} concentration was observed (Fig. 3), and 7 mM Mg^{++} caused a 40% fall in activity. The concentrations of MgAMP and uncomplexed AMP were calculated as functions of the Mg^{++} concentration, and showed that at 10.3 mM Mg^{++} 33% of the total AMP was present in the uncomplexed form.[5] A plot of reaction velocity against free AMP was also sigmoidal, indicating that the inhibition was not due simply to depletion of AMP by complexation with Mg^{++} and suggesting that MgAMP may be an inhibitor or a very poor substrate.

Song and Bodansky (13) found that the 5′-nucleotidase of rat liver plasma membrane had optimal activity in the absence of Mg^{++} at pH 7.5, and exhibited a second optimum at pH 9.1–9.3 in the presence of Mg^{++}. Similar behavior of the rat heart enzyme could explain the difference in the pH optimum found in this work and that of 9.5 reported by Baer *et al.* (8), whose studies were carried out in the presence of Mg^{++}. Low concentrations of Mg^{++} activated both the rat liver and rat heart enzymes (13, 8), while the enzyme purified from sheep brain and that present in rat heart homogenate were unaffected by Mg^{++} (7, 14). The variation in the effects of Mg^{++} on 5′-nucleotidase reported by different investigators may be due to Mg^{++}-induced shifts in the pH optima of the reactions. Similarly, the sigmoidal dependence of the inhibition of 5′-nucleotidase activity on Mg^{++} concentration reported here may be related to change in the pH optimum with increasing Mg^{++} concentration as well as to possible inhibition by MgAMP. Calcium ions inhibited rat heart 5′-nucleotidase more strongly than Mg^{++}, but in a typical hyperbolic manner (Fig. 3), and 2 mM Ca^{++} caused 40% inhibition of activity. The striking difference in the plots of enzyme activity with respect to

[5] Calculated by Dr. W. J. O'Sullivan.

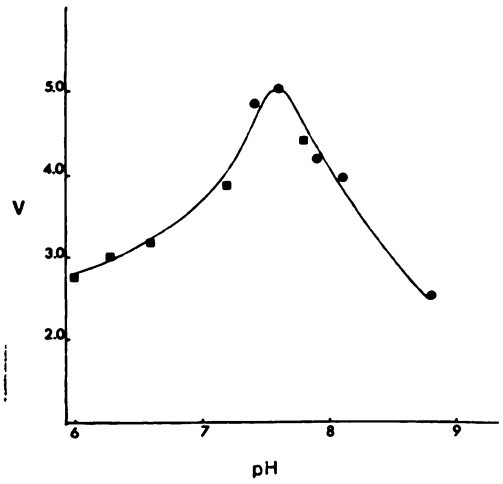

FIG. 2. *Effect of pH on 5′-nucleotidase activity*

The spectrophotometric assay was used with 48 μM AMP and the appropriate buffer. Each assay contained 36 units of 5′-nucleotidase, and the initial velocity, V, is expressed as nanomoles of AMP hydrolyzed per minute per 36 units of enzyme. ■, 0.05 M potassium phosphate buffer; ●, 0.05 M Tris-HCl buffer. At pH 7.6 V was the same in each buffer.

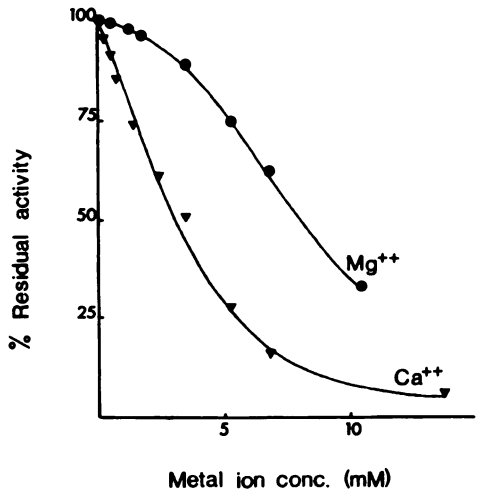

FIG. 3. *Effect of magnesium and calcium ions on 5′-nucleotidase activity*

Reaction velocity was measured by the spectrophotometric assay as described in MATERIALS AND METHODS, using 55 μM AMP and 220 μg of enzyme protein in each assay. One hundred per cent activity was 6.1 mμmoles of AMP hydrolyzed per minute per 220 μg of protein.

Mg^{++} and Ca^{++} concentrations (Fig. 3) indicates that the cations inhibit by different mechanisms.

Substrate Specificity

The response of 5′-nucleotidase activity to AMP concentration was hyperbolic, and a double-reciprocal plot of the initial velocity data obtained using the spectrophotometric assay gave a K_m of 1.65×10^{-5} M and a V_{max} of 106 mμmoles hydrolyzed/min/mg of protein. Similar K_m and V_{max} values for AMP were obtained when rates were measured using the colorimetric assay. AMPS, 2-chloro-AMP, 2-methylthio-AMP, IMP, and GMP were substrates, and all showed a hyperbolic dependence of reaction velocity on substrate concentration. Accurate K_m and V_{max} values with standard errors were computed for the six substrates by Wilkinson's weighted nonlinear regression method (15). Comparison of these parameters (Table 2) shows that the different substrates have similar affinities for the enzyme, but that the enzyme-substrate complex is broken down at different rates, depending both on the nature of the substituents on the

TABLE 2

Kinetic constants of substrates of rat heart 5′-nucleotidase

Enzyme activity was assayed by measurement of the release of inorganic phosphate as described under MATERIALS AND METHODS. Assays contained 34 μg of enzyme protein and were done in duplicate. With AMP as substrate, activity was also assayed by the spectrophotometric method as described in the legend to Fig. 4. Parameters were calculated by Wilkinson's weighted nonlinear regression method (15).

Substrate	K_m	V_{max}
	$M \times 10^5$	*mμmoles/min/mg*
AMP	1.65 ± 0.10[a]	106.4 ± 1.8[a]
AMP	1.45 ± 0.06	103.9 ± 1.0
IMP	1.23 ± 0.06	56.4 ± 0.8
GMP	1.17 ± 0.08	33.6 ± 0.5
2-Chloro-AMP	1.25 ± 0.07	82.3 ± 1.0
2-Methylthio-AMP	2.01 ± 0.11	120.8 ± 2.0
AMPS	1.14 ± 0.10	37.8 ± 0.8

[a] These parameters were obtained by the spectrophotometric assay.

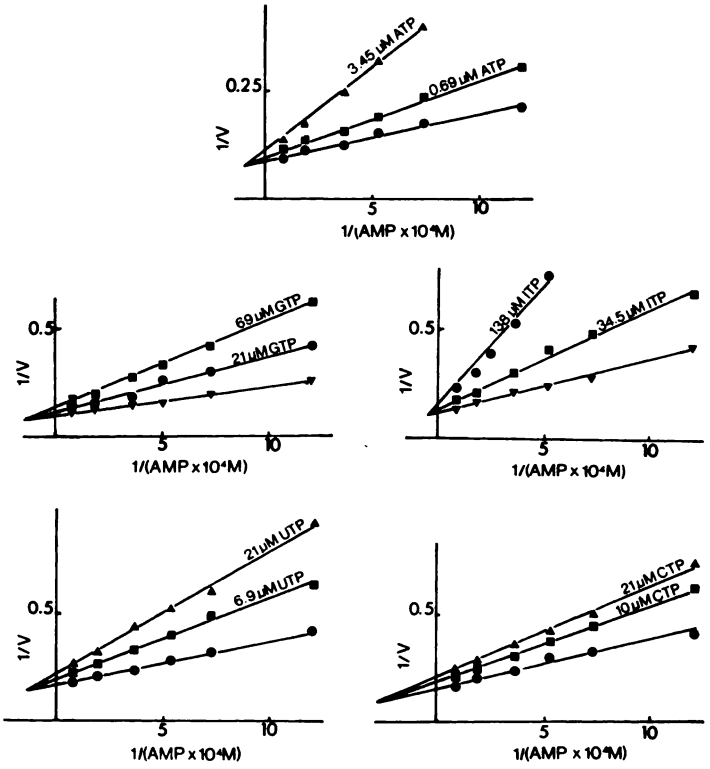

FIG. 4. *Inhibition of 5′-nucleotidase by nucleoside triphosphates*

The reciprocals of the initial velocity with respect to the reciprocal of AMP concentration were plotted in the absence and in the presence of ATP, GTP, ITP, UTP, and CTP. Velocity (V) is expressed as nanomoles of AMP hydrolyzed per minute per 120 μg of protein. The spectrophotometric assay as described in MATERIALS AND METHODS was used. Assays contained 120 μg of enzyme protein, and were performed in triplicate.

purine ring and on the nature of the acidic moiety. Thus AMPS and GMP are hydrolyzed at 36% and 32%, respectively, IMP at 54%, and 2-chloro-AMP at 79% of the rate of AMP. 2-Methylthio-AMP, in contrast, is hydrolyzed more rapidly than AMP. Murray and Atkinson (9) found that the 5′-nucleotidase of *Crotalus adamanteus* hydrolyzed AMPS to adenosine at only 1.9% of the rate of AMP, indicating that the snake venom enzyme differs significantly in its active site requirements from the mammalian heart enzyme.

Inhibition by Nucleoside Triphosphates

Rat heart 5′-nucleotidase was strongly inhibited by ATP and UTP; CTP, GTP, and ITP also inhibited, but less strongly. In each case the inhibition was of the mixed competitive-noncompetitive type (Fig. 4). Inhibition constants were obtained from replots and are summarized in Table 3. Inhibition of mammalian 5′-nucleotidases by nucleoside triphosphates has been reported. Baer *et al.* (8) found that ATP in the presence of Mg^{++} was a competitive inhibitor of partially purified rat heart 5′-nucleotidase, with a K_i of 1.83×10^{-6} M, and that GTP and ITP were also inhibitors. The sheep brain enzyme was found by Ipata (7) to be inhibited by ATP, UTP, and CTP, but not GTP, and the inhibition curves obtained with increasing concentrations of ATP, UTP, and CTP were sigmoidal. In contrast, inhibition of the rat heart enzyme by increasing concentrations of ATP, UTP, CTP, GTP, and ITP was noncooperative (Fig. 5).

TABLE 3

Inhibition constants for nucleoside triphosphate inhibitors of 5′-nucleotidase

Inhibitor constants were obtained from slope against inhibitor concentration replots of the data shown in Fig. 4. Details of the assays are given in the legend to Fig. 4.

Nucleoside triphosphate	K_i
	$M \times 10^6$
Adenosine 5′-triphosphate	1.60
Guanosine 5′-triphosphate	34.5
Inosine 5′-triphosphate	41.0
Cytidine 5′-triphosphate	14.0
Uridine 5′-triphosphate	12.3

DISCUSSION

Both AMP and AMPS are hydrolyzed by 5′-nucleotidase to produce adenosine, a vasodilator which is about equipotent with AMP and, like AMP, has a transient effect (1). Adenosine is rapidly removed from the circulation by uptake by tissues and erythrocytes and by deamination to inosine (16, 17). The lower rate of dephosphorylation of AMPS compared to AMP suggests that the greater duration of action of AMPS *in vivo* may be related to its slower hydrolysis to adenosine by 5′-nucleotidase. 2-Chloro-AMP, which is 6–7 times as potent as AMP (1), is hydrolyzed by 5′-nucleotidase at 80% of the AMP rate to give 2-chloroadenosine, an extremely potent and long-lasting vasodilator. 2-Methylthio-AMP, which is without measurable vasodilator properties, is hydrolyzed at a greater rate than AMP to give 2-methylthioadenosine, a weak but long-lasting vasodilator. The ratio of vasodilator potencies of adenosine, 2-chloroadenosine, and 2-methylthioadenosine in the rat is 1:8:0.1, respectively (1, 2). Thus there is a correlation between the vasodilator potencies and duration of action of AMP, AMPS, 2-chloro-AMP, and 2-methylthio-AMP and the potencies and duration of action of the nucleosides produced from them by hydrolysis by 5′-nucleotidase.

It is not known whether AMP acts directly as a vasodilator *in vivo* or acts after hydrolysis to adenosine, as suggested by Jacob and Berne (18). Baer and Drummond (19) showed that AMP on a single perfusion through the isolated rat heart was converted substantially to adenosine, indicating that 5′-nucleotidase is present in the walls of the coronary vessels. The histochemical studies of Bajusz and Jasmin on the rat heart (20) located 5′-nucleotidase activity in the walls of the coronary vessels. Thus, during passage through the cardiovascular system, AMP and its vasoactive analogues may be readily hydrolyzed to the parent nucleosides by 5′-nucleotidase in the coronary vessels, but whether plasma phosphatases also play a role in the hydrolysis of AMP and the AMP analogues *in vivo* remains to be determined.

The inhibition of the rat heart 5′-nucleotidase by both purine and pyrimidine triphosphates indicates that the requirements of the enzyme binding site, or sites, for the triphosphates are somewhat nonspecific. Like the sheep brain enzyme (7), the heart enzyme is powerfully inhibited by ATP, which has a K_i of 1.6×10^{-6} M. However, the

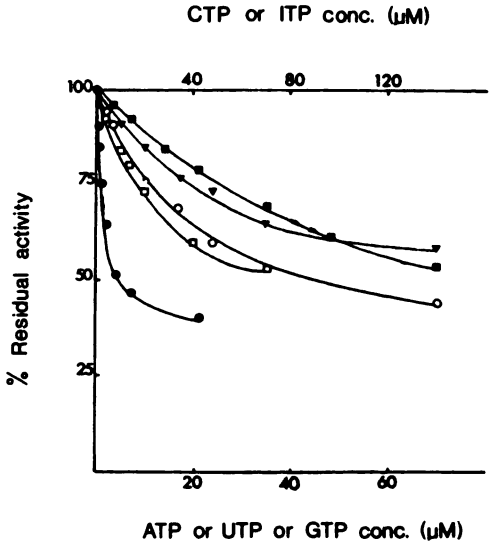

FIG. 5. *Effect of varying concentrations of ATP* (●), *UTP* (□), *GTP* (■), *CTP* (○), *and ITP* (▼) *on the reaction velocity of 5′-nucleotidase*

The spectrophotometric method of assay as described in MATERIALS AND METHODS was used with 55 μM AMP; each assay contained 120 μg of enzyme protein, which in the absence of nucleoside triphosphate hydrolyzed 9.3 mμmoles of AMP per minute.

two enzymes differ in their response to GTP, which is an inhibitor of the heart enzyme with a K_i of 3.46×10^{-5} M but does not affect the activity of the brain 5′-nucleotidase (7). Burger and Lowenstein (21) showed that the two pathways for AMP breakdown in the rat heart are regulated by ATP and GTP. They demonstrated that ATP stimulated the formation of IMP from AMP and inhibited the dephosphorylation of AMP, and that GTP reversed the activation of adenylate deaminase by ATP and also inhibited the formation of adenosine from AMP. This dual control regulates AMP metabolism according to the requirements of the myocardium for the intermediates of the two pathways; i.e., for IMP, which is needed for guanosine nucleotide synthesis, and for adenosine, whose release by the myocardium regulates coronary blood flow (22).

ATP levels in rat heart are normally high, 4 μmoles/g of tissue, and fall substantially only on prolonged ischemia (23). ATP is such a powerful inhibitor of 5′-nucleotidase that, even allowing for its concentration within certain cellular compartments, normal conditions would appear to be inhibitory for the enzyme, preventing the formation of adenosine from AMP. However, 60% of the 5′-nucleotidase activity of rat heart homogenate is found in the membrane-derived microsomal fraction together with 30–40% of the Mg^{++}-activated ATPase activity.[6] Hydrolysis of ATP by Mg^{++}-ATPase in the vicinity of 5′-nucleotidase may cause a localized fall in the ATP level, so that adenosine can be produced from AMP by 5′-nucleotidase and released to regulate coronary blood flow.

ACKNOWLEDGMENT

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[6] M. H. Maguire and S. Steggall, unpublished observations.

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