Formation and Deamination of Adenosine by Cardiac Muscle Enzymes

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SUMMARY

The enzymic catabolism of adenylic acid has been studied in rat heart. AMP may be degraded by two routes, one involving dephosphorylation to adenosine followed by deamination to inosine, the other involving deamination to inosinic acid followed by dephosphorylation to inosine. The data are consistent with the role of adenosine as a regulator of coronary flow. The 5′-nucleotidase and adenosine deaminase have been isolated and partially purified from rat heart acetone powder. The deaminase has also been partially purified from red blood cells. ATP is a potent competitive inhibitor of 5′-nucleotidase and could function as a regulator for the production of adenosine. The substrate specificity of adenosine deaminase has been examined. Several adenosine analogs function as competitive inhibitors of both the heart and red cell enzyme. The most potent inhibitors tested were N^6-methyladenosine and 6-methoxypurine ribonucleoside. The coronary dilator, Persantin, does not inhibit cardiac adenosine deaminase at concentrations as high as 4×10^{-4} M.

INTRODUCTION

A present concept of the autoregulation of coronary flow is that adenosine constitutes the physiological mediating substance. Careful analysis of the products of nucleotide breakdown in rat hearts (1) and rabbit hearts (2) have shown that during hypoxia substantial amounts of adenosine are formed in the myocardium. Inosine and hypoxanthine are also present (2, 3). It has long been known that adenosine is a potent coronary dilator (4–6), whereas inosine, its deamination product, is inactive. Gerlach and associates (1) have concluded that the main pathway of adenine nucleotide degradation in heart muscle proceeds via AMP → adenosine → inosine → hypoxanthine. This course is different from that in other tissues, such as skeletal muscle (2), where AMP is deaminated to IMP prior to dephosphorylation. From such observations Berne (3) has postulated that, during myocardial hypoxia, adenosine is formed from the degradation of cardiac adenine nucleotides, diffuses out of the myocardial cells, reaches the coronary arterioles via the interstitial fluid and produces coronary dilation, thus counteracting the oxygen deficiency. Considerable evidence has been published which suggests that the coronary dilator, Persantin,[1] acts by altering adenosine catabolism. Berne [see Bunag *et al.* (7)] has reported that this drug inhibits cardiac adenosine deaminase. Kübler and Bretschneider (8) have shown that this agent prevents adenosine deamination in intact dog erythrocytes, but the effect disappears when the cells are

[1] 2,6-Bis(diethylamino)-4,8-dipiperidino-pyrimido-(5,4-*d*)-pyrimidine. We are indebted to Dr. G. H. Worsley, Boehringer Ingelheim Products, Montreal, P. Q., for a sample of this compound.

lysed. They have provided evidence that Persantin does not inhibit deaminase but prevents the transport of adenosine across cellular membranes.

Very little is known concerning the properties of the enzymes in cardiac tissue and blood which catalyze the conversion of AMP to adenosine and inosine. Such information might be of value for a complete understanding of the physiological importance of these catabolic events in regulating blood flow to the heart. The present report is concerned with the identification and properties of a 5′-nucleotidase in cardiac tissue together with a study of the properties of adenosine deaminase in ventricular tissue and red blood cells. The presence of adenylic deaminase in cardiac tissue is also established by direct enzymic test.

MATERIALS AND METHODS

Materials

Adenosine deaminase from calf intestinal mucosa was purchased from Sigma Chemical Company. The ribonucleosides of 6-chloropurine, 6-mercaptopurine, 6-methylmercaptopurine, 6-benzylmercaptopurine, 2-amino-6-mercaptopurine, 2-amino-6-methoxypurine, 2-amino-6-chloropurine, 2-amino-6-methylmercaptopurine, 2,6-diaminopurine, and N^6-methylaminopurine, as well as 2-fluoroadenosine, psicofuranosyladenine, 3′-deoxy-3′-aminoadenosine, and 6-amino-9-D-ribofuranosyl-7-desazapurine (tubercidin) were kindly provided by Dr. Ian C. Caldwell, McEachern Laboratory, Edmonton, Alberta. All other compounds were obtained from commercial sources.

General Methods

Activity of 5′-nucleotidase in crude extracts was measured by incubating 12.5 μmoles Tris-HCl, pH 8.5, 2 μmoles $MgCl_2$, 5 μmoles IMP, and extract in a total volume of 0.25 ml at 37°. The reaction was stopped after 30 min by the addition of 0.2 ml of 12% trichloroacetic acid and 0.25 ml water. After centrifugation an aliquot was assayed for inorganic phosphate by the method of Fiske and SubbaRow (9). Activity in preparations which were free of adenylic deaminase were assayed by incubating 50 μmoles glycylglycine buffer pH 8.7, 5 μmoles AMP, 4 μmoles $MgCl_2$, and enzyme in a total volume of 0.5 ml at 37°. The reaction was stopped after 30 min by the addition of trichloroacetic acid. With sufficiently pure preparations the molybdate–sulfuric acid reagent was added directly to the incubation mixture to stop the reaction. One unit of 5′-nucleotidase activity is expressed as the amount of enzyme that catalyzed the liberation of 0.1 μmole of inorganic phosphate under these conditions. The purified enzyme was conveniently assayed optically by coupling with excess adenosine deaminase. This assay mixture contained 150 μmoles Tris-HCl pH 7.5, 4 μmoles $MgCl_2$, excess calf intestinal mucosa adenosine deaminase,[2] AMP, and other additions in a final volume of 1.5 ml. The reaction was started by addition of the nucleotidase, and the decrease in absorbance at 265 mμ was followed at 30-sec intervals for 5 min ($d = 0.5$ cm) in a Model DU spectrophotometer with photomultiplier attachment, temperature 28–30°.

Adenosine deaminase was assayed by incubating 1.25 μmoles adenosine in 0.1 M citrate pH 6.5, together with heart extract, in a final volume of 1.5 ml. The decrease in absorbance at 265 mμ was followed at 30-sec intervals ($d = 0.5$ cm, temperature = 28–30°). One unit of activity is that amount of enzyme which catalyzed the deamination of 0.1 μmole of adenosine per minute, determined from initial velocities. Specific activity is defined as units per milligram protein. Protein was determined by the method of Lowry *et al.* (10), the biuret method (11), or optically by the procedure of Warburg and Christian (12).

Cellular fractionation. Rat hearts were perfused by the Langendorf technique to remove blood. The ventricular tissue was blotted, weighed, and homogenized (Potter-Elvehjem homogenizer) in 10 volumes of

[2] The commercial enzyme was dialyzed against 10 mM Tris-HCl, pH 7.0, before use. An amount of enzyme sufficient to deaminate 0.5 μmole of adenosine per minute, based on initial velocities, was used in the assay system.

0.25 M sucrose. The homogenate was strained through cheesecloth and centrifuged at 300 *g* at 0° for 10 min in a Servall refrigerated centrifuge. The precipitate was washed twice by suspending in 4 volumes of 0.25 M sucrose, homogenizing briefly, and centrifuging. The supernatant fluids were added to the first 300 *g* supernatant solution. The nuclear fraction was suspended in 0.25 M sucrose. Thereafter mitochondrial, microsomal, and soluble fractions were obtained by standard procedures. The final 100,000 *g* supernatant fluid was placed in dialysis casing and concentrated to one-eighth the original volume by immersion in aquacide (Calbiochem). Nitrogen was determined by nesslerimetry.

Isolation of Enzymes

Hearts were removed from twenty rats and perfused with Tyrode's solution by the Langendorf technique for 2 min to thoroughly remove blood. The ventricles were cut out, blotted, and weighed (wt 11.8 g). The minced ventricles were homogenized in 10 volumes of acetone at —15° using a Servall Omni-Mixer. After centrifugation at —15° the precipitate was extracted twice more with 10 volumes of acetone in a similar manner. The final residue was thoroughly dried *in vacuo* to yield 2 g of powder.

5'-Nucleotidase. The acetone powder was extracted with a final volume of 60 ml of 50 mM Tris-HCl, pH 7.5, by suspending first in a chilled glass mortar, then by thorough homogenizing in a loose-fitting Potter-Elvehjem homogenizer. The suspension was centrifuged at 30,000 *g* for 15 min. The supernatant fluid (55 ml) was kept for isolation of adenosine deaminase. The precipitate was extracted once more with 25 ml of buffer, followed by centrifugation. This supernatant fluid was discarded. The precipitate was suspended by homogenizing in 20 ml of 50 mM Tris-HCl, pH 7.5, containing 0.15 M KCl. To this well-stirred solution 3 ml of buffer containing 230 mg of sodium deoxycholate was added. The mixture was kept at 4° for 4 hr, then centrifuged at 30,000 *g* for 20 min. The precipitate was suspended in 3 ml of buffer containing 1% deoxycholate and centrifuged again. The combined supernatants were centrifuged at 100,000 *g* for 1 hr. The recovered supernatant fluid was stirred at 4°, and 6 ml of cold distilled *n*-butanol was added. After 5 min the mixture was centrifuged at 3,000 *g* for 20 min. The aqueous phase was carefully removed and thoroughly dialyzed against several changes of 50 mM Tris-HCl, pH 7.5, containing 0.15 M KCl. After dialysis sodium deoxycholate was added to achieve a final concentration of 0.5%, and the solution was centrifuged at 30,000 *g* for 15 min to remove cloudiness. The nucleotidase was then precipitated by fractionating between 40 and 60% saturation with ammonium sulfate which was added from a saturated solution at 4°. The resulting precipitate was dissolved in 2.5 ml of buffer containing 0.15 M KCl and 0.5% deoxycholate. The final preparation of nucleotidase was stable at 4° for weeks and could be repeatedly frozen and thawed without loss in activity.

Adenosine deaminase. The supernatant fluid from the extraction of the acetone powder (55 ml) (see above) was brought to 0.15 M with respect to KCl, adjusted to pH 6.8 and brought to 60% saturation with ammonium sulfate by the addition of 20.3 *g* of the solid salt at 4°, the pH being maintained at 6.8 by the addition of KOH. The mixture was stirred 15 min and centrifuged at 30,000 *g*. The precipitate (which contained all the adenylic deaminase of the starting fraction) was routinely discarded. The supernatant fluid was brought to 80% saturation by the addition of 8.3 *g* of ammonium sulfate as before. After centrifugation, the precipitate was dissolved in 20 mM Tris-HCl, pH 7.5, and dialyzed 6 hr against 3 liters of this buffer (final volume 4.5 ml). To this solution was added one-quarter volume of 1 M potassium citrate, pH 6.0, followed by one-eighth volume of 1 M zinc acetate. After stirring at 4° for 10 min, ethanol was added dropwise to a final concentration of 14% by volume while the temperature was lowered to —6°. After 10 min the mixture was centrifuged at —6°. Ethanol was added to the supernatant fluid to a final concentration of 31% by

volume while the temperature was lowered to —10°. After centrifugation the precipitate was suspended in 50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 0.1% glutathione. The preparation was dialyzed initially against 1 liter of 20 mM Tris-HCl containing 5 mM EDTA and finally for 12 hr against 3 liters of 20 mM Tris-HCl, pH 7.5 (final volume 1.7 ml). The enzyme at this stage was completely free of 5′-nucleotidase and adenylic acid deaminase. It could be repeatedly frozen and thawed without loss of activity.

Adenosine deaminase from red blood cells. Heparinized rat blood (55 ml) was centrifuged at 3000 rpm for 20 min. The plasma was carefully removed and frozen. The packed red cells were washed twice with heparinized saline, and were lysed by the addition of 4 volumes of water followed by homogenization. The deaminase was precipitated between 55 and 80% saturation with ammonium sulfate, which was added in the solid form at pH 6.8. The precipitate was dissolved in 20 mM Tris-HCl, pH 7.5, and dialyzed against 3 liters of this buffer; the final volume was 7.5 ml. The enzyme was then precipitated from solution by fractionating between 14 and 31% ethanol in the presence of Zn^{2+} ion in a manner identical to that for the heart enzyme. To the dialyzed fraction, volume 1.6 ml, was added 0.2 ml of 1 M succinate, pH 6.0, and the solution was placed in a water bath at 57° for 2½ min. After chilling, denatured protein was removed by centrifugation. The colorless solution was stored in the frozen state.

RESULTS

Preliminary. Previous investigators (1–3) have shown that adenosine is not present in detectable amounts in the perfusion fluid or in the myocardium of well oxygenated perfused mammalian hearts, nor can it be identified in the coronary sinus blood of anesthetized dogs. Inosine and hypoxanthine, however, are readily detectable in these experimental preparations and could arise via dephosphorylation of IMP. Adenosine appears only under conditions of anoxia (1, 2). To examine the nature of nucleotide catabolism more directly, fresh rat heart homogenate was incubated with AMP (1.5 μmoles in a final volume of 0.2 ml) at pH 7.5 and 37°. After 10 and 30 min the reaction was stopped by the addition of 0.02 ml of glacial acetic acid, and 0.025 ml of the reaction mixture was subjected to descending chromatography in isopropanol–ammonia–water (6:3:1).

The chromatograms revealed that AMP was extensively degraded, IMP and inosine being the only products present. Inosine could have been formed exclusively from dephosphorylation of IMP by the action of adenylic deaminase. When, however, incubations were carried out at pH 9.0 (where adenylic deaminase is inactive) the products of the reaction were adenosine and inosine. No IMP was detectable. This indicated that rat heart extracts were indeed capable of dephosphorylating AMP to adenosine. These preliminary experiments provided evidence that rat heart is capable of degrading AMP by two routes, one by dephosphorylation to adenosine with subsequent deamination to inosine, or secondly by deamination to IMP followed by dephosphorylation of this compound to inosine. The first pathway would require the concerted action of 5′-nucleotidase and adenosine deaminase, the second being catalyzed by the action of adenylic acid deaminase and 5′-nucleotidase.

Cellular distribution of the catabolic enzymes. The cellular distribution of the three enzymes involved in AMP catabolism is shown in Table 1. It is evident that a major portion of the nucleotidase is located in the microsomal and nuclear fractions, in agreement with Bajusz and Jasarin (13), who provided histochemical evidence that a membrane-bound nucleotidase was present in rat heart. Adenosine deaminase is found exclusively in the soluble cytoplasmic fraction.

Studies on 5′-Nucleotidase

A survey was made of nucleotidase activity in hearts of other species, with the hope that ventricular tissue from larger animals might prove to be a better source of the enzyme. Table 2 reveals that there

TABLE 1
Cellular distribution of 5'-nucleotidase, adenylic deaminase, and adenosine deaminase

Cell fraction	Per cent total nitrogen	5'-Nucleotidase		Adenylic deaminase		Adenosine deaminase	
		Total activity (units)	Per cent of total activity	Total activity (units)	Per cent of total activity	Total activity (units)	Per cent of total activity
Whole homogenate	100	5,500	100	8.28	100	18.25	100
Nuclear fraction	42.2	1,980	34	4.1	51	ND[a]	—
Mitochondria	19.3	495	9	0.42	5.6	ND	—
Microsomes	7.2	2,200	40	0.77	10.2	ND	—
Soluble supernatant	20.8	935	17	2.49	33.4	17.7	97

[a] ND, no detectable activity. Rat hearts were fractionated as described in the text.

TABLE 2
Activity of 5'-nucleotidase in hearts of various species

Whole homogenates were used. Results were calculated on the basis of units per gram of fresh tissue and expressed as activity relative to that of rat heart (1000 units/g) taken as 100. 5'-IMP was used as the substrate.

Species	Relative activity
Rat	100
Guinea pig	33
Mouse	17
Sheep	16.5
Beef	3.0
Pig	1.5
Rabbit	1.0

TABLE 3
Purification of rat heart 5'-nucleotidase

Fraction	Total units	Specific activity (units/mg protein)	Yield of activity (%)
Suspension of acetone powder residue[a]	8,800	—	—
Deoxycholate-treated extract	14,500	21	"100"
100,000 *g* supernatant	13,900	43	94
n-Butanol fraction	6,500	81	45
40–60% saturated ammonium sulfate	2,900	260	22

[a] Residue obtained after extracting soluble protein with dilute buffer.

is a surprising variation in activity of this enzyme in hearts of those species examined. Rat hearts were the most active and were chosen for further study.

The nucleotidase was not rendered soluble during the preparation of an acetone powder and therefore it could be readily separated from adenosine deaminase which was quantitatively solubilized by extracting an acetone powder with dilute buffer. The level of purification of the nucleotidase is shown in Table 3. A full solubilization of the enzyme could be achieved by deoxycholate treatment of the residue remaining after extraction of the acetone powder with dilute buffer. This is evidenced by an almost quantitative yield of the enzyme obtained in the supernatant after centrifuging the deoxycholate-treated extract at 100,000 *g*. Sodium deoxycholate treatment also led to an apparent increase in enzyme units. Although the purification achieved is not great, the enzyme was free of adenylic deaminase, adenosine deaminase, myokinase, and ATPase, and was suitable for kinetic studies. The enzyme was activated 30% by Mg^{2+} ion at a concentration of 7 mM in the assay and inhibited 50% by this concentration of Ca^{2+} ion when assayed with AMP at pH 8.7. The purified nucleotidase was not specific for AMP, but hydrolyzed a variety of nucleoside 5'-monophosphates (Table 4). The preparation hydrolyzed glucose 6-phosphate and 2'(3')-AMP at 1.5 and 2.7% of the rate of AMP, respectively, indicating a small contamination of alkaline phosphatase.

Effect of pH on nucleotidase activity. The nucleotidase was active over a broad

TABLE 4
Hydrolysis of phosphate esters by heart 5'-nucleotidase

Substrate	Relative rate
5'-AMP	100
5'-UMP	106
5'-CMP	120
5'-IMP	65
5'-GMP	59
5'-d-AMP	50
ATP	0
Glucose-6-PO_4	1.5
2'(3')-AMP[a]	2.7

[a] 2'(3')-AMP was tested using the optical assay.

range of pH (Fig. 1) with an optimal activity at pH 9.5. When assayed in the presence of glycine buffer, activity did not fall off above pH 9.5, but remained optimal at pH values as high as 10.8.

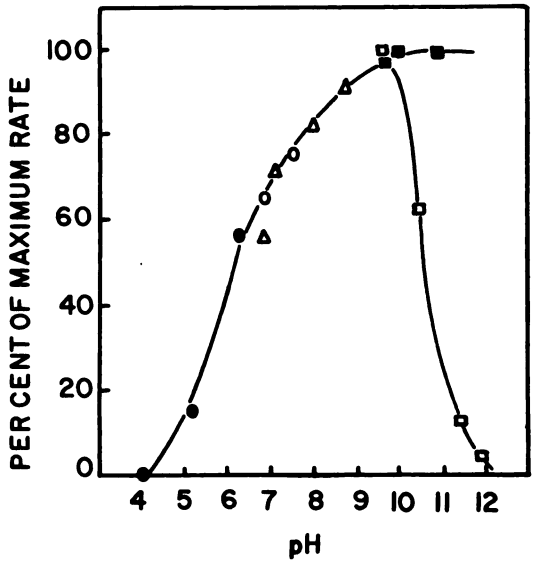

FIG. 1. *Effect of pH on 5'-nucleotidase activity*
The standard assay, based on inorganic phosphate liberation, was used and the pH varied: —●— acetate; —○— Tris-HCl; —△— glycylglycine; —■— glycine; —□— lysine.

Effect of substrate concentration and ATP inhibition. Attempts to determine the K_m of the enzyme, using the assay for inorganic phosphate, were complicated by the low sensitivity of the method. However, the optical assay coupled with excess adenosine deaminase served well for this purpose. From the Lineweaver-Burke plot (Fig. 2) a K_m of 1.8×10^{-5} M was calculated.

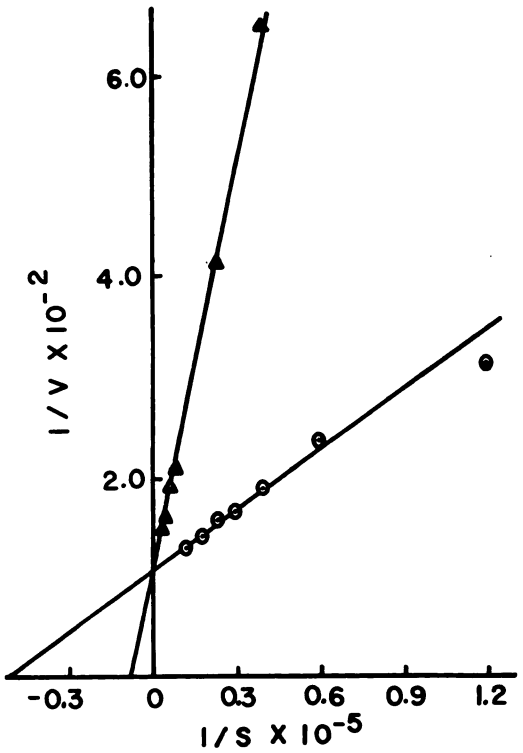

FIG. 2. *Double-reciprocal plots for heart 5'-nucleotidase and its inhibition by ATP*
The optical assay as described in the text was used. Initial velocities were defined in terms of μmoles of substrate hydrolyzed per minute: —○— AMP alone; —△—, AMP in presence of 1.33×10^{-5} M ATP.

It was found that ATP was a powerful competitive inhibitor of the nucleotidase. From the double reciprocal plot (Fig. 2) a K_i of 1.83×10^{-6} M was calculated. GTP and ITP also inhibited the nucleotidase and were one-tenth and one-twentieth as effective, respectively, as ATP.

Studies on Adenosine Deaminase

On the basis of the assay of whole homogenates, it can be calculated that rat hearts can deaminate adenosine at the rate of 0.81 μmole per gram of fresh tissue per

minute at 25°. The activity of adenylic deaminase is such that AMP is deaminated at the rate of 0.30 μmole/g·min. The purification procedure described for cardiac adenosine deaminase yielded an increase in specific activity from 0.05 for the suspension of the acetone powder to 0.44 for the 60–80% ammonium sulfate fraction, and this was increased in the final 14–31% ethanol fraction to 2.0, representing a 40-fold purification. The overall yield was 55%. The final preparation was free of 5′-nucleotidase and adenylic deaminase. Rat plasma deaminates adenosine at the very low rate of 0.015 μmole/min·ml based on the conditions of the optical assay. A rate of 0.025 μmole/min·ml of packed red cells was calculated which is equivalent to 0.012 μmole/min by red cells present in 1 ml of blood. The purification procedure for the red cell deaminase led to a final preparation (heated fraction) with a specific activity of 0.48, representing a 750-fold enrichment over the lysed red cells with a yield of 60%.

Effect of pH on cardiac adenosine deaminase. The enzyme was active over a broad range of pH between 5 and 9, with an optimum between pH 6.5 and 7. Activity at pH 5.5 was 80% of maximum and at pH 8.0 was 70% of maximum. The enzyme was equally active in citrate, phosphate, and Tris-HCl buffers.

Substrates and inhibitors. The K_m of the cardiac deaminase for adenosine was calculated from Lineweaver-Burke plots to be 7.2×10^{-5} M. The value obtained for the purified red cell enzyme was 3.9×10^{-5} M. The relative rates of deamination of a number of adenosine analogs are shown in Table 5. Both enzymes displayed a similar substrate specificity. The purified cardiac enzyme preparation dechlorinated 6-chloropurine ribonucleoside and 2-amino-6-chloropurine ribonucleoside. The absorption spectrum of the latter compound before and after incubation with the enzyme is shown in Fig. 3. The product is clearly guanosine. Similarly, the product of dechlorination of 6-chloropurine ribonucleoside was shown to be inosine. The initial velocities of the reaction could be measured by following the increase in absorbance at 250 mμ. In addition, dechlorination of 2-amino-6-chloropurine ribonucleoside could be measured by the decrease in absorbance at 310 mμ. In this way, it was established that the ratio of the rate of the reaction with respect to adenosine was 0.11 for 6-chloropurine ribonucleoside and 0.58 for 2-amino-6-chloropurine ribonucleoside (Table 5). The latter compound is dechlorinated about 1.6 times faster than its amino analog, 2,6-diaminopurine ribonucleoside, is deaminated. The product of deamination of the latter compound was shown to be guanosine. While this work was in progress a paper appeared by Cory and Suhadolnik (14) in which they showed that intestinal mucosa adenosine deaminase dechlorinates 6-chloropurine ribonucleoside to yield inosine, with a velocity ratio to adenosine deamination of 0.1.

TABLE 5
Substrate specificity of adenosine deaminase

Compound[a]	Heart deaminase relative initial velocity	Red cell deaminase relative initial velocity
Adenosine	1.0	1.0
2′-Deoxyadenosine	1.1	1.2
Adenine	0	—
5′-Adenylic acid	0	—
2′-Adenylic acid	0	—
2′,3′-Cyclic adenylic acid	0	—
3′-Deoxy-3′-amino-adenosine	0.16	0.16
Isopropylidine adenosine	0.38	—
2,6-Diaminopurine ribonucleoside	0.37	—
4′-Thioadenosine	0.51	0.50
Tubercidin	0	—
2-Fluoroadenosine	0	0
Psicofuranosyl adenosine	0	0
Adenylyl adenosine (ApA)	0	—
6-Chloropurine ribonucleoside	0.11	—
2-Amino-6-chloropurine ribonucleoside	0.58	—

[a] All compounds were present in the assay at 8.4×10^{-5} M.

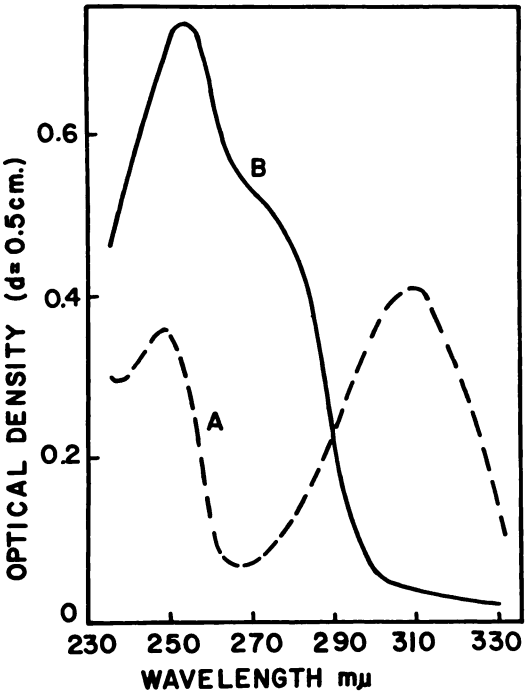

FIG. 3. *Action of purified rat heart adenosine deaminase on 2-amino-6-chloropurine ribonucleoside*

The compound was present at 1.3×10^{-4} M. Absorption spectra were taken before adding enzyme (curve *A*) and after the reaction had reached completion (curve *B*).

A variety of adenosine analogs acted as competitive inhibitors of both the cardiac and red cell deaminase (Table 6). K_i values for several of these were calculated from double-reciprocal plots. N^6-methyladenosine was the most potent inhibitor, giving a K_i of 1.2×10^{-5} M and 1.3×10^{-5} M for the heart and red cell enzyme, respectively. By means of the direct optical assay it was also established that Persantin did not inhibit the heart deaminase at concentrations as high as 4×10^{-4} M, which is in agreement with the findings of Kübler and Bretschneider (8). In order to test this compound, the assays had to be conducted at pH 5.2 because of the very low solubility of this substance at higher pH values.

DISCUSSION

The data provide direct enzymic evidence that two routes of AMP catabolism are available to the heart. This nucleotide can be dephosphorylated to adenosine; the latter compound can then be deaminated to inosine. Adenylic deaminase is also present so that AMP degradation can proceed via deamination to IMP, which is then converted to inosine by dephosphorylation. This is in agreement with Gerlach *et al.* (1), who came to similar conclusions based on analysis of nucleotide intermediates from perchloric acid extracts of ischemic heart tissue. The findings are also consistent with the idea that adenosine may function as a regulator of coronary flow since the enzymic machinery for its formation and degradation is clearly present. Why adenosine is present only in anoxic tissue is not exactly clear. On a tissue weight basis the nucleotidase and adenosine deaminase have about equal activity. Adenylic deaminase is about half as active as adenosine deaminase on a tissue weight basis. In normal heart tissue, inosine is probably formed both from dephosphorylation of IMP and by deamination of adenosine, with the result that adenosine is kept below detectable levels. During hypoxia the formation of adenosine must either be accelerated or its destruction inhibited. It might be that ATP controls adenosine formation. Since ATP is a potent inhibitor of the nucleotidase, one might speculate that in well oxygenated tissue the nucleotidase is maintained in an inhibited state by the high levels of this nucleotide. During hypoxia, when ATP levels fall sharply (2), the nucleotidase may thus be activated with resulting increase in adenosine levels. An argument against this reasoning, however, comes from the very low K_i value of ATP as an inhibitor. It might be difficult to conceive the levels of this nucleotide falling low enough to permit activation of the enzyme. It must be remembered, however, that the nucleotidase is membrane bound, and the concentrations of ATP existing at the site might be considerably lower than those in other areas of the cell. Thus a lowering of ATP at the site of the nucleotidase during hypoxia may well serve to activate the enzyme. The fact that the nucleotidase is particulate may

TABLE 6
Inhibitors of adenosine[a] deaminase

Compound	Concentration ($\times 10^4$ M)	Inhibition (%)	K_i ($\times 10^5$ M)
	Heart deaminase		
6-Mercaptopurine ribonucleoside	1.7	41	—
6-Methylmercaptopurine ribonucleoside	4.2	15	—
6-Benzylmercaptopurine ribonucleoside	4.2	8	—
2-Amino-6-mercaptopurine ribonucleoside	4.2	50	—
2-Amino-6-methylmercaptopurine ribonucleoside	4.2	12	—
6-Methoxypurine ribonucleoside	0.79	31	10
2-Amino-6-methoxypurine ribonucleoside	0.84	37	—
2-Amino-6-methoxypurine ribonucleoside	4.2	87	—
N^6-Methyladenosine	0.17	41	—
N^6-Methyladenosine	0.34	60	1.2
2-Fluoroadenosine	2.0	31	—
2-Fluoroadenosine	4.0	62	—
8-Azaguanine	2.5	12	—
8-Azaguanine	10.0	48	—
	Red cell deaminase		
N^6-Methyladenosine	0.17	49	1.3
6-Methoxypurine ribonucleoside	0.79	41	4.1
6-Mercaptopurine ribonucleoside	1.66	52	6.8

[a] Adenosine was present at 8.4×10^{-5} M.

also serve to allow adenosine to escape from the cell without being deaminated in the soluble cytoplasm. Whether the enzymes for AMP catabolism are present in the smooth muscle of the coronary vasculature is not known. At any rate, adenosine crosses cellular barriers readily and if it indeed enters the coronaries to produce dilation it could readily be deaminated either by diffusing back into the cardiac cell or by the deaminase present in plasma and red cells. Although the level of this enzyme is low in plasma and red cells, the large volume of blood flowing through the coronaries (60 ml/min per 100 g for dog hearts) might still provide an adequate deamination mechanism.

Several competitive inhibitors of adenosine deaminase have been described, of which N^6-methyladenosine and 6-methoxypurine ribonucleoside are the most potent. The possibility exists that these structural analogs may increase coronary flow by inhibiting adenosine deaminase. Some of them might well be coronary dilators in themselves. Several reports have appeared describing the cardiovascular action of 2-chloroadenosine (15, 16) and recently Large and Wein (17) have shown that this compound is 50–250 times more potent as a coronary dilator than adenosine and its action lasted much longer. The structural requirements for coronary dilator effects could readily be examined with the analogs used here.

The study of different adenosine analogs as inhibitors or substrates for the deaminase of heart and red cells has led to essentially the same results as those for the intestinal mucosa enzyme published by Cory and Suhadolnik (14, 18) while this manuscript was in preparation. Of particular interest is the finding that the enzyme has a deaminating and a dechlorinating action, which suggests that the basic function of the deaminase is to facilitate or mediate a nucleophilic displacement at the 6-position of the adenosine system. In the case of deamination, an additional function should be an activation by protonating the 6-amino group. Potent nucleophilic substituents like mercapto or methylmercapto groups are not displaced. The fact that 2-fluoroadenosine acts as an inhibitor rather

than a substrate may be the result of the function of such a substituent in aromatic systems in reducing nucleophilic attack. The less potent amino substituent in the 2-position still permits displacement at the 6-position as shown by the conversion of 2,6-diaminopurine ribonucleoside and 2-amino-6-chloropurine ribonucleoside to guanosine. A comparison of the relative rates of dechlorination and deamination of the compounds, 2-amino-6-chloropurine ribonucleoside, 6-chloropurine ribonucleoside, and 2,6-diaminopurine ribonucleoside and adenosine, respectively, casts some doubt on this interpretation; the interaction of the enzyme and substrate may be more complicated. Since the results reported by Cory and Suhadolnik (14, 18) are in quantitative agreement with our findings, it is assumed that no other enzymes are involved in the dechlorination and in the action on the 2-amino derivatives. In this connection it is interesting to note the close similarity in the properties of the deaminase of different animal and tissue sources.

The findings of Schaeffer and Vince (19), that bulky groups at the 6-position lower the inhibtory action, is supported by the fact that 6-methylmercaptopurine ribonucleoside is a less effective inhibitor than the corresponding mercapto compound. The absence of inhibitory function of tubercidin indicates that the N^7-position is probably an essential binding site (18), assuming that the steric change is not significant. Extensive consideration of the effects of alterations in the sugar moiety is not possible on the basis of the present data, although it is apparent that charged groups (phosphate) and polar groups such as the 3′-amino (as in 3′-deoxy-3′-aminoadenosine) completely prevent or strongly reduce binding to the enzyme.

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