Studies on Adenosine Deaminase

I. Purification and Properties of Ox Heart Adenosine Deaminase

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SUMMARY

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4.) has been purified 1060-fold from ox heart muscle. Adenosine, 2-deoxyadenosine, 2,6-diaminopurineriboside, 6-hydroxylaminopurineriboside and 6-chloropurine riboside are substrates of the enzyme, and adenosine and deoxyadenosine both exhibit substrate inhibition at concentrations of substrate about five times the Michaelis value. A number of 2-substituted adenosine analogs that have vasodilator properties have been shown to inhibit the enzyme competitively, and the cardiac glycoside ouabain has been found to be a competitive inhibitor. N^6-methylation of adenosine and of several 2-substituted adenosines gave inhibitors with increased affinity for the enzyme active site; however, N^6-dimethyl adenosine and adenosine-1-*N*-oxide inhibited noncompetitively. The relationship between the structure of the cardioactive adenosine analogs and their affinity for adenosine deaminase is considered.

INTRODUCTION

Adenosine and its phosphorylated derivatives, AMP, ADP, and ATP, have been known for many years to have potent effects on the mammalian heart and vascular system, eliciting bradycardia, heart block, and coronary vasodilatation (1, 2). Berne (3) has suggested that adenosine may play an important part in regulating coronary blood flow. The response of the mammalian heart to adenosine has been found to be species dependent; thus guinea pig heart is very sensitive to small doses of adenosine, the main effect being one of auriculoventricular block, whereas cat and rabbit heart are not so sensitive (2).

These responses to adenosine are transient, probably because of the rapid deamination of adenosine by adenosine deaminase to inactive inosine. Rand *et al.* (4), in this department, demonstrated that the auriculoventricular block caused by adenosine in guinea pig heart could be increased up to 10 times by ouabain in doses of ouabain which were otherwise without effect on guinea pig heart, and they suggested that this may be due to the inhibition of cardiac adenosine deaminase by ouabain. Several workers (5, 6) have attempted to demonstrate this inhibition using rat and guinea pig heart homogenates, but with equivocal results.

2-Chloroadenosine, which was shown by Clarke *et al.* (7) to cause prolonged vasodilatation in the cat does not cause heart block in the cat on intraatrial injection (F. Michal, personal communication). Thorp and Cobbin (8) showed that 2-chloroadenosine has 10 times the potency of adenosine in causing heart block on intraatrial injection in the guinea pig, and that it has a prolonged effect which is not potentiated by ouabain. These authors suggested that the prolonged effect was due to nondeamination of the analog by cardiac adenosine deaminase.

It is possible that the species difference in the response of the mammalian heart to

adenosine and 2-substituted adenosines is related to the properties of the particular cardiac adenosine deaminase, and a comparative study of cardiac adenosine deaminases has been commenced with the purification of the enzyme from ox heart. This study incorporates particularly the examination of the effects on the cardiac adenosine deaminase of adenosine analogs having vasodilatatory activity.

Adenosine deaminase has not been previously purified from mammalian heart; however after the present work had been completed, a paper by Baer *et al.* (9) was published in which some properties of the partially purified rat heart adenosine deaminase were reported. Adenosine deaminase from calf intestinal mucosa has been highly purified by Brady and O'Connell (10) and found to have a specific activity of 430 enzyme units (at 37°) and to be homogeneous in the ultracentrifuge. A number of workers (11, 12) have studied the effects of nucleoside analogs on partially purified preparations of calf intestinal adenosine deaminase with a view toward elucidating the binding requirements of the enzyme.

MATERIALS AND METHODS

All adenosine analogs with the exception of 2,6-diaminopurineriboside, adenosine-1-*N*-oxide, and 6-hydroxylaminopurineriboside were synthesized in this department. Adenosine-1-*N*-oxide and 6-hydroxylaminopurineriboside were gifts from Dr. G. B. Brown and Dr. A. Giner-Sorolla of the Sloan-Kettering Institute for Cancer Research, New York; 2,6-diaminopurineriboside was a Fluka product. N^6-methyladenosine (13) and N^6-dimethyladenosine (14) were prepared from 6-chloropurineriboside by reaction with aqueous methylamine and dimethylamine, respectively. 2-Methylthioadenosine and 2-methoxyadenosine were both prepared from 2-chloroadenosine as described by Schaeffer and Thomas (15). 2-Chloroadenosine was synthesized either by a modification of the procedure of Brown and Weliky (16), or via fusion of 2,6-dichloropurine with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribose.[1] The preparation of 2-trifluoromethyladenosine has been recently published (17), and syntheses of N^6-methylated 2-chloro-, 2-methylthio-, and 2-trifluoromethyladenosines will be reported in a separate communication.[1] Deoxyinosine and guanosine were obtained from Light and Co., and inosine and deoxyadenosine from Fluka.

Ammonium sulfate was B.D.H. enzyme grade, β,β′-dimethylglutaric acid was purchased from Fluka, and bovine serum albumin fraction V was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. DEAE-cellulose was Whatman Powder DE 50, and CM cellulose was Whatman Powder CM 70. DEAE-Sephadex A-50 was obtained from Pharmacia, Sweden.

Ox hearts were obtained within 3 min of killing; the coronary vessels were perfused with 2% sodium citrate, and blood was washed from the chambers with 0.9% saline. The hearts were then packed in ice for transport to the laboratory, where they were stored at −10°.

Assays. The buffer used in all assays and kinetic measurements, with the exception of the pH/activity curve, was sodium β,β′-dimethylglutarate (18), which has negligible ultraviolet absorption from 280 to 240 mμ.

Enzyme purification was followed by the Conway microdiffusion method (19) of estimation of the ammonia liberated when the enzyme preparation (1 ml) was incubated for 15 min with adenosine (15 μmole) in 0.1 M buffer (1 ml) at pH 6.8 and 25°. The deaminase reaction was stopped by pipetting an aliquot of the incubation mixture directly into concentrated KOH contained in the Conway unit. Assays were done in duplicate.

The activity of column eluates was measured spectrophotometrically by Kalckar's method (20), following the decrease in optical density at 265 mμ resulting from the conversion of adenosine to inosine. The eluate (0.25 ml) was added to a 1-cm cuvette containing 0.3 μmole adenosine in 2.75 ml of 0.05 M buffer pH 7.

[1] G. Gough and M. H. Maguire, in preparation.

Kinetic measurements. These were carried out using a Perkin-Elmer 350 spectrophotometer fitted with a time drive and cuvettes thermostatted at 25°. Initial rates of deamination of adenosine and deoxyadenosine by adenosine deaminase were determined by recording the fall in O.D. at 265 mμ immediately after addition of the enzyme to the substrate in 0.05 M buffer at pH 7.0. The blank cuvette contained buffer only. Deamination of 10 μg/ml of adenosine in a cuvette of 1 cm light path results in a fall in O.D. of 0.263 units; deamination of 10 μg/ml of deoxyadenosine results in a fall of 0.280 O.D. units. The initial rate of deamination of 2,6-diaminopurineriboside was similarly measured by recording the increase in O.D. at 244 mμ. Deamination of 10 μg/ml of 2,6-diaminopurineriboside in a 1 cm cuvette gives an increase in O.D. of 0.15 O.D. units at 244 mμ. Beer's law was found to hold in 0.5 cm cuvettes in the range of 0–80 μg of adenosine per milliliter of buffer, in 1.0 cm cuvettes in the range 0–30 μg/ml and in 2.0 cm cells 0–15 μg/ml. Measurements were made in concentration ranges in which Beer's law held. Cuvettes of 0.5 cm light path were used for V_{max} and K_m determinations; the enzyme (0.05 ml of a solution having 0.346 enzyme units/ml) was added to the substrate in 1.45 ml of 0.05 M buffer. The blank cuvette contained buffer only. For inhibition studies cells of 2.0 cm light path were used; the enzyme (0.1 ml of a solution having 0.346 enzyme units/ml) was added to adenosine and the inhibitor in 5.9 ml of 0.05 M buffer. The blank cuvette contained the same concentration of inhibitor in buffer. All rate measurements were done in triplicate, and the mean values were used to plot data.

Enzyme unit. One unit of activity is defined as the amount of enzyme which will catalyze the deamination of 1 μmole of adenosine per minute at 25° and at an adenosine concentration of 10×10^{-5} M.

Protein assay. Protein was determined colorimetrically by the method of Lowry *et al.* (21) using bovine serum albumin fraction V in the range of 0–50 μg as a standard. The relative protein concentration of column eluates was estimated by measuring the O.D. at 280 mμ.

Purification of adenosine deaminase. All the following operations were carried out at 2°.

One hundred grams of frozen heart tissue were homogenized in a blendor-mix with

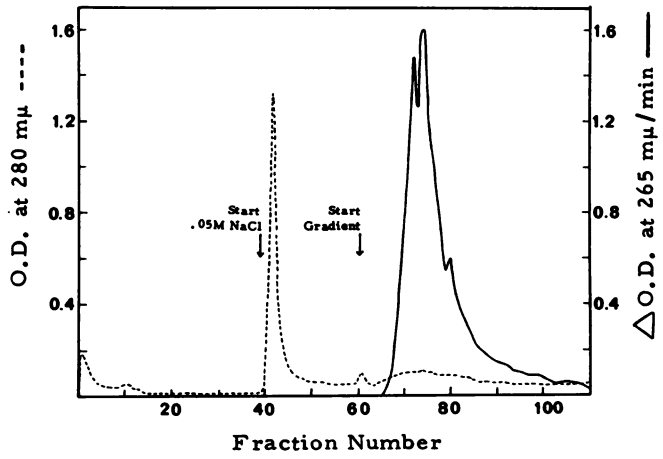

FIG. 1. *DEAE-cellulose column chromatography of adenosine deaminase*

Thirty-four milligrams of protein was introduced onto the column in 250 ml 0.005 M Tris buffer pH 7. A buffer wash was followed by elution with 0.05 M NaCl in buffer. Adenosine deaminase was eluted with a gradient of 0.05 to 0.2 M NaCl in buffer. Then 10-ml fractions were collected and enzyme activity (——) was assayed spectrophotometrically as described under Materials and Methods. The relative protein concentration (---) of fractions was obtained by measurement of the optical density at 280 mμ.

1000 ml distilled water for 3 min. Centrifugation of this suspension for 1 hour at 6000 *g* gave a clear pink supernatant. This was made 0.2 M with respect to $MgSO_4$, 0.005 M with respect to sodium acetate, and the pH was adjusted to 6.5. Acetone at −10° was added slowly to the cooled (0°) stirred solution to a final acetone concentration of 44% (v/v). The precipitate which formed was allowed to flocculate for 10 min and was centrifuged for 30 min at −6° and 7000 *g* to give a clear red supernatant. Acetone was similarly added to the supernatant to a final acetone concentration of 63% (v/v). The colored protein precipitate was spun down at −6° and 7000 *g* and dissolved in 200 ml 0.1 mM EDTA. The solution was dialyzed overnight against distilled water and fractionated by addition of solid ammonium sulfate (29.7 g/100 ml) to give a 50% saturated solution. The suspension obtained was allowed to stand for 10 minutes and centrifuged for 30 min at 9000 *g* to give a clear red supernatant. Addition of ammonium sulfate to this solution to 75% saturation precipitated active colored protein, which was centrifuged as before and dissolved in 50 ml 0.1 mM EDTA. The extraction to this stage was repeated three times, and the 4 aqueous solutions of active protein were combined and dialyzed for 2 days against several changes of cold water, then overnight against 0.005 M citrate buffer pH 5.0 containing 1 mM 2-mercaptoethanol. All buffers from this step on contained 1 mM 2-mercaptoethanol. The pink dialyzed solution (240 ml) was then run onto a 1.5 g CM-cellulose column (1 × 20 cm) packed in 0.005 M citrate, and washed through with 10 ml of buffer. The first 5 ml of eluate was discarded, and the remainder was collected in one fraction which contained 87% of the activity loaded onto the column. Colored protein remained on the column. The eluate was dialyzed overnight against 0.005 M Tris pH 7.0, and was introduced onto a 1.5 g DEAE-cellulose column (1 × 20 cm) packed by gravity in the same buffer. The column was washed first with the buffer, then with 0.05 M NaCl in buffer followed by a gradient of 0.05 M to 0.2 M NaCl (the constant volume reservoir contained initially 100 ml of 0.05 M NaCl in buffer); 10 ml fractions were collected. Adenosine deaminase activity was eluted by the gradient, and recovery of activity from the column was 78% (Fig. 1). The active fractions were then pooled and dialyzed overnight against 0.01 M citrate buffer pH 6.0. The enzyme solution was concentrated to 85 ml by ultrafiltration using the technique of Everall and Wright (22), and introduced onto a 1 g DEAE-Sephadex A-50 column (1 × 12 cm) which was prepared by gravity packing in the same buffer. A 15 ml buffer wash was followed by stepwise elution with NaCl in

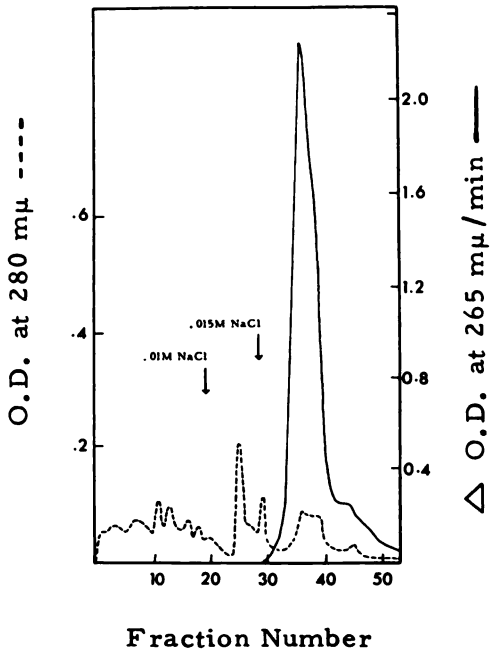

FIG. 2. *DEAE-Sephadex column chromatography of adenosine deaminase*

Six milligrams of protein was put on the column in 85 ml 0.01 M citrate buffer pH 6.0. A buffer wash was followed by stepwise elution with increasing concentrations of NaCl in buffer. Then 5-ml fractions were collected, and enzyme activity (——) was assayed spectrophotometrically as described under Materials and Methods. The relative protein concentration (----) was obtained by measurement of the optical density at 280 mμ. Adenosine deaminase was eluted at 0.015 *M* NaCl.

TABLE 1
Purification of adenosine deaminase from ox heart muscle

Fraction	Volume (ml)	Activity[a] (units/ml)	Protein (mg/ml)	Specific[a] activity (units/mg)	Yield (%)	Purification
Aqueous extract	3990	2.3	2.05	1.12	100	—
44–63% acetone precipitate	1037	6.7	2.12	3.16	76	2.8
50–75% $(NH_4)_2SO_4$ precipitate	262.5	19.2	0.86	22.25	55	20
CM-cellulose eluate	248	16.2	0.14	115.6	44	103
DEAE-cellulose eluate	200	17.8	0.045	396.0	39	354
DEAE-Sephadex A-50 eluate	38	42.8	0.036	1190	18	1060

[a] The unit of activity here is the amount of enzyme which will deaminate 1 μmole of adenosine in 15 min at 25°, as described under Materials and Methods.

buffer; 5-ml fractions were collected. 0.015 M NaCl eluted adenosine deaminase as a single sharp peak (Fig. 2). The active fractions were pooled and represented a 71% recovery of activity from the column and 1060-fold purification of adenosine deaminase in an overall yield of 18%. Table 1 contains a summary of the purification procedure.

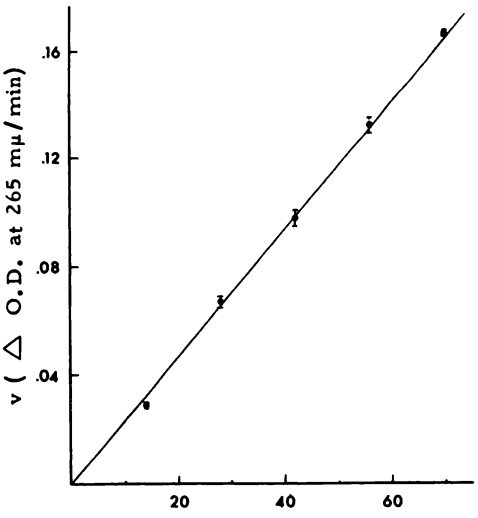

FIG. 3. *Variation of the rate of deamination of adenosine with adenosine deaminase concentration*

Adenosine concentration was 10×10^{-5} M; 1-cm cuvettes were used, and the assay method is described under Materials and Methods.

RESULTS

General Properties of Adenosine Deaminase from Ox Heart

The purified cardiac adenosine deaminase had a specific activity of 103 enzyme units and retained 80% of activity on storage at 2–4° for 3 months at pH 6.0 in citrate buffer.

At constant adenosine concentration the velocity of deamination by adenosine deaminase increased linearly with enzyme concentration (Fig. 3).

The pH optimum for the deamination of adenosine by cardiac adenosine deaminase in 0.05 M phosphate buffer was found to be 7.0 (see Fig. 4), and the pH activity curve closely resembles that found by Brady and O'Connell (10) for purified adenosine deaminase from calf intestinal mucosa. Coddington (23) however found a pH optimum of 7.6 for a partially purified preparation of the calf mucosal enzyme.

Substrate Specificity

Adenosine, deoxyadenosine, and 2,6-diaminopurineriboside were found to be substrates of cardiac adenosine deaminase.

Adenosine and deoxyadenosine both exhibited substrate inhibition at concentrations of substrate above 17.5×10^{-5} M, but 2,6-diaminopurineriboside did not inhibit the enzyme at concentrations up to 30.75×10^{-5} M (see Fig. 5). Double reciprocal plots of the initial velocity data were used to obtain K_m and V_{max} values. Lines were fitted to the plotted data by eye, and in

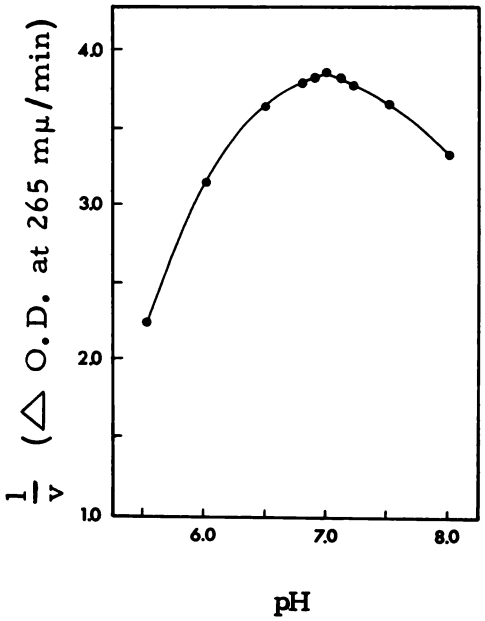

FIG. 4. *pH Activity curve of ox heart adenosine deaminase in 0.05 M phosphate buffer*

One-centimeter cuvettes were used. The sample cuvette contained 0.1 ml enzyme (approximately 0.07 unit), 32 μg adenosine and 2.9 ml 0.05 M phosphate buffer at the required pH. Initial velocities were measured as described under Materials and Methods.

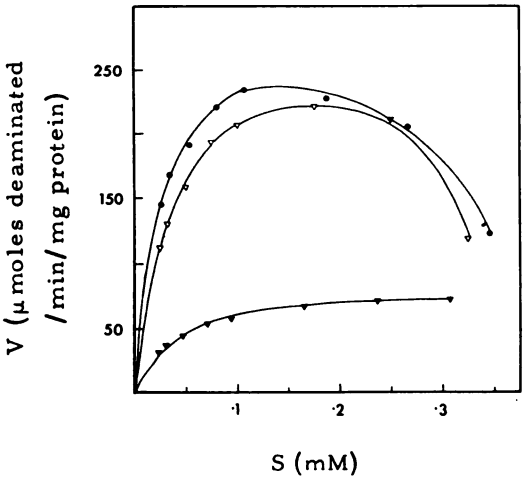

FIG. 5. *Variation of velocity with substrate concentration*

▽, adenosine; ●, deoxyadenosine; ▼, 2,6-diaminopurineriboside. Initial velocities were measured as described under Materials and Methods.

the cases of adenosine and deoxyadenosine it was necessary to extrapolate the linear part of the slope to intercept the ordinate and absisca (see Figs. 6 and 7). The values

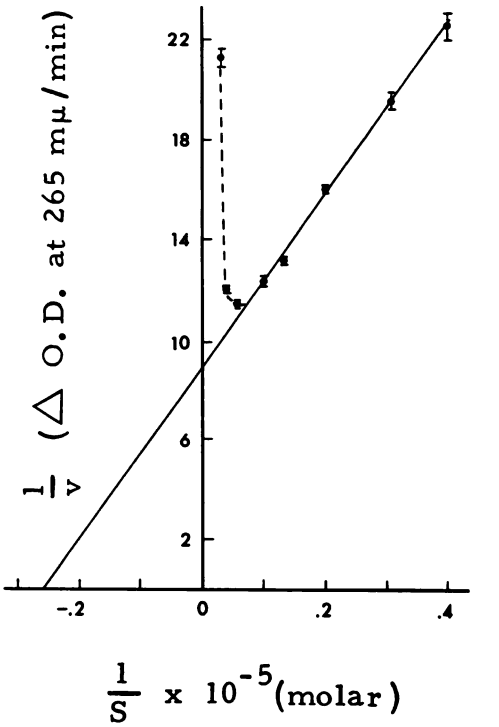

FIG. 6. *Double reciprocal plot of adenosine concentration against initial velocity of the deaminase reaction*

Assay conditions are described under Materials and Methods.

obtained by this method are given in parentheses in Table 2. To obtain accurate values for K_m and V_{max} Wilkinson's weighted nonlinear regression method (24) for computing these parameters and the relevant standard errors was used. A computer program was written, and the data were processed by the KDF 9 computer to give the parameters and their standard errors; these are recorded in Table 2.

These parameters show that 2,6-diaminopurineriboside has much the same affinity for the active site as does adenosine, but is deaminated at one-third the rate of the other two substrates.

6-Hydroxylaminopurineriboside was found to be a substrate of ox heart adenosine

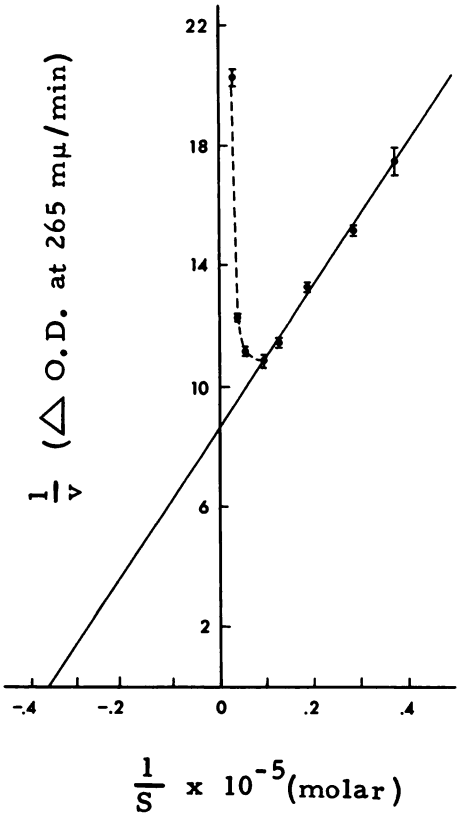

FIG. 7. *Double reciprocal plot of deoxyadenosine concentration against initial velocity of the deaminase reaction*

Assay conditions are described under Materials and Methods.

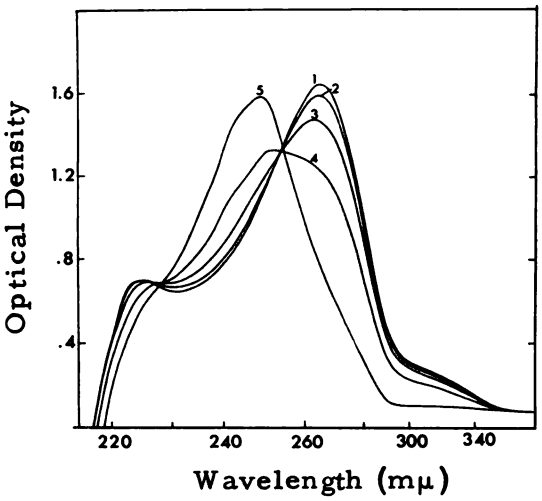

FIG. 8. *Ultraviolet spectral changes with time obtained when 6-hydroxylaminopurineriboside was incubated with adenosine deaminase*

The enzyme (0.7 unit in 0.1 ml) was added to 6-hydroxylaminopurineriboside (90 μg) in 3 ml 0.05 M buffer in a 1-cm cuvette, and the spectrum was scanned after 0.5 min (curve *2*), 5 min (curve *3*), 15 min (curve *4*), and 90 min (curve *5*). Curve *1* is the spectrum of 6-hydroxylaminopurineriboside prior to the addition of the enzyme, and curve *5* is the spectrum of the product, inosine.

deaminase and under standard assay conditions was slowly converted to inosine (see Fig. 8). The kinetics of dehydroxylamination are being investigated at present. Ox heart adenosine deaminase was also found to hydrolyze 6-chloropurineriboside to give inosine; hydrolysis of 6-chloropurineriboside to inosine is a known property of both partially purified rat heart and calf intestinal mucosa adenosine deaminases (9, 12).

Ten analogs of adenosine have been shown to inhibit cardiac adenosine deaminase. Inhibition studies were carried out by measuring the initial velocity of the deaminase reaction in the presence and absence of inhibitors as described under

TABLE 2
Kinetic constants for adenosine deaminase

Substrate	K_m[a] (M × 10^5)	V_{max}[a] (μmole/min/mg protein)
Adenosine	4.05 ± 0.29 (3.85)	291.6 ± 8.7 (282)
Deoxyadenosine	2.69 ± 0.18 (2.74)	293.4 ± 6.4 (295)
2,6-Diaminopurineriboside	3.77 ± 0.13 (3.92)	82.4 ± 6.9 (83)

[a] The values in parentheses are those obtained directly from Lineweaver-Burk plots.

Materials and Methods. Inhibitor constants were calculated from the relationship

$$K_i = \frac{i}{\left(\frac{K_p}{K_m} - 1\right)}$$

using the appropriate intercepts of Lineweaver-Burk plots to obtain K_m and K_p (25). In each case the nature of the inhibition was verified by plotting the velocity data by the methods of Hanes (26) and Hofstee (27).

N^6-Methyladenosine, 2-chloro-, 2-methoxy-, 2-methylthio-, and 2-trifluoromethyladenosines, also 2-chloro-N^6-methyl-, 2-methylthio-N^6-methyl-, and 2-trifluoromethyl-N^6-methyladenosines were all found to be competitive inhibitors. The K_i's obtained are listed in Table 3. Figure 9 shows some plots typical of those obtained.

DISCUSSION

Substrate inhibition and product inhibition of adenosine deaminase have not been previously reported. The concentration levels of adenosine and inosine required to inhibit ox heart adenosine deaminase are relatively high (adenosine inhibits above 1.75×10^{-4} M and inosine has a K_i of 3.59×10^{-4} M), and it seems unlikely that these properties play a role in the regulation of heart function. In the guinea pig for example, the adenosine concentration found to cause toxic heart block effects is about 10^{-6} M (4), much lower than that required to inhibit ox heart adenosine deaminase.

Ouabain has now been shown to inhibit a cardiac adenosine deaminase, but the concentration level required for inhibition (10^{-3} M) is much higher than the amount of ouabain (10^{-7} M) which potentiates adenosine bradycardia in the guinea pig (4). Although there may be species differences between ox heart and guinea pig heart adenosine deaminase with regard to affinity for ouabain, it would appear that the potentiating effect of ouabain on the adenosine heart block is not due to the inhibition of cardiac adenosine deaminase. Moreover it has not been possible to demonstrate a potentiation of adenosine heart block in the guinea pig by the powerful adenosine

TABLE 3

Compounds found to inhibit ox heart adenosine deaminase

Inhibitor	K_i (M $\times 10^5$)	Nature of inhibition
N^6-Methyladenosine	0.60	Competitive
2-Chloroadenosine	5.70	Competitive
2-Methoxyadenosine	4.78	Competitive
2-Methylthioadenosine	3.19	Competitive
2-Trifluoromethyladenosine	9.97	Competitive
2-Chloro-N^6-methyladenosine	0.37	Competitive
2-Methylthio-N^6-methyladenosine	0.19	Competitive
2-Trifluoromethyl-N^6-methyladenosine	0.24	Competitive
Inosine	35.9	Competitive
Guanosine	8.94	Competitive
Ouabain	741.0	Competitive
Adenosine 1-N-oxide	—	Noncompetitive
N^6-Dimethyladenosine	—	Noncompetitive

N^6-Dimethyladenosine at 3.39×10^{-5} M and adenosine-1-N-oxide at 5.3×10^{-5} M were both found to inhibit noncompetitively. At a concentration of 1.37×10^{-3} M ouabain proved to be a weak competitive inhibitor of the deamination of adenosine, with a K_i of 7.41×10^{-3} M.

Both inosine and guanosine, the products of deamination of adenosine and of 2,6-diaminopurineriboside, were found to inhibit the enzyme competitively; guanosine is the stronger inhibitor (see Table 3).

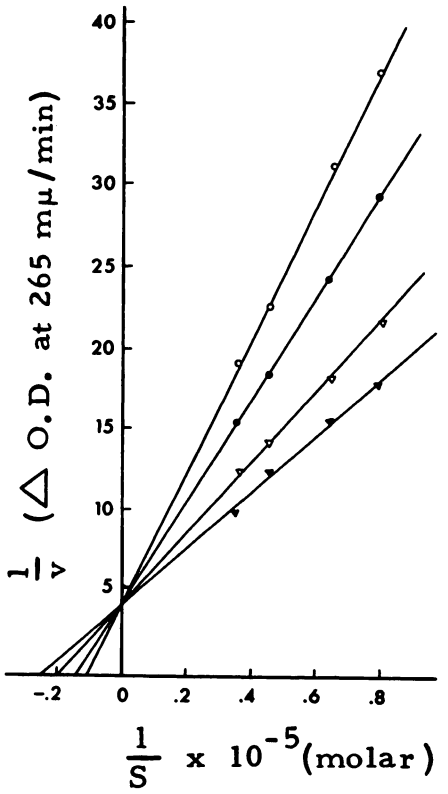

FIG. 9. *Double reciprocal plots of adenosine alone and in the presence of inhibitors, versus initial velocity of the deaminase reaction*

▼, Adenosine alone; ▽, adenosine plus 2.99 × 10^{-6} M 2-trifluoromethyladenosine; ●, adenosine plus 3.17 × 10^{-6} M 2-chloro-N^6-methyladenosine; ○, adenosine plus 8.90 × 10^{-6} M N^6-methyladenosine.

deaminase inhibitor 2-chloro-N^6-methyladenosine (F. Michal, personal communication).

2-Chloroadenosine, which has 10 times the potency of adenosine in causing heart block in the guinea pig and has long-lasting effects (8), was not deaminated by ox heart adenosine deaminase and was found to be a competitive inhibitor of the enzyme. It seems likely that the duration of the toxic heart effects of this analog is due to its nondeamination by cardiac adenosine deaminase.

The 2-substituted adenosine analogs with a free 6-amino group which inhibit ox heart adenosine deaminase (see Table 3) have been found to cause prolonged vasodilatation in a number of mammalian species (7, 8). The N^6-methylated analogs also cause prolonged vasodilatation in the cat and the guinea pig, and the order of potency of the vasodilatatory effect relative to adenosine in the cat has been found to be 2-chloroadenosine 10, adenosine 1, 2-chloro-N^6-methyladenosine 0.5, 2-methoxyadenosine 0.2, 2-trifluoromethyladenosine and 2-methylthioadenosine 0.1, 2-trifluoromethyl-N^6-methyladenosine 0.05, and 2-methylthio-N^6-methyladenosine < 0.05. (F. Michal, personal communication).

Comparison of the relative potencies of these analogs with their inhibitor constants (see Table 3) shows that there is no correlation between the inhibitory effects of the analogs on ox heart adenosine deaminase and their efficacy as vasodilatatory agents.

The affinities of the analogs for the active site of adenosine deaminase relative to the affinity of adenosine can be compared if one assumes that the K_s values for adenosine and 2,6-diaminopurineriboside equal the K_m values. In Table 4 the affinities of the analogs relative to adenosine are tabulated.

TABLE 4

Affinities[a] of analogs for the active site of adenosine deaminase relative to adenosine

Compound	Relative affinity of adenosine analog	Relative affinity of N^6-methylated adenosine analog
Adenosine	1.0	6.7
2,6-Diaminopurineriboside	1.08	—
2-Methoxyadenosine	0.85	—
2-Methylthioadenosine	1.27	21.4
2-Chloroadenosine	0.78	10.9
2-Trifluoromethyladenosine	0.41	16.9

[a] Affinities were calculated as reciprocals of the K_m or K_i values.

2,6-Diaminopurineriboside is seen to have much the same affinity as adenosine, while 2-trifluoromethyladenosine has less than half this affinity. The affinities of the 2-substituted adenosines appear to depend

mainly on the electronegativity of the substituent group. pKa values for these analogs are not available yet, but the base-weakening or base-strengthening effects of the substituents may be expected to follow the effects exerted by these groups when substituted in the pyrimidine ring of purine. Albert and Brown (28) have measured the following basic pKa values: purine 2.39, 6-aminopurine 4.22, 6-methoxypurine 2.21, 6-methylthiopurine 0, 6-chloropurine < 2, and 6-trifluoromethylpurine < 0. The trend of both the acid and basic pKa values found by Albert and Brown for these compounds shows that the basic pKa for 6-chloropurine may be expected to fall between those of 6-methylthio- and 6-trifluoromethylpurine. The order of increasing electronegativity of the substituents is then amino (the only base-strengthening substituent), <methoxy, <methylthio, <chloro-, and <trifluoromethyl. This order mirrors a decrease in affinity of the analogous substituted adenosines for the active site of adenosine deaminase, with one exception, 2-methylthioadenosine, which has a base-weakening substituent yet has a greater affinity for the active site than does adenosine. The anomaly cannot be explained on a steric basis as the methylthio group is one of the largest of these substituents and might be expected to prevent a good "fit" of 2-methylthioadenosine in the active site. 2,6-Diaminopurineriboside on the other hand has a substituent that is both small and electron-donating, and "fit" in the active site is such that aminohydrolysis can occur.

The correlation between the electron-withdrawing effect of the 2-substituent and the affinity of the adenosine analog for the active site suggests that the electron density of the pyrimidine ring of the analog, particularly the electron density of N_1, may play an important role in binding the analog to a specific group in the active site. N_1 has been shown by Jones and Robins (29) to be the most basic nitrogen of adenosine.

The N^6-monomethylated adenosine analogs all have greater affinity for the active site than the parent adenosines (Table 4), N^6-methyl-2-trifluoromethyladenosine, for example, has 41 times the affinity of 2-trifluoromethyladenosine. While these increases could be due to hydrophobic bonding of the N^6-methyl group with a suitable hydrophobic area in the active site, they may well be due to increased basicity of the pyrimidine ring, particularly of N_1, due to hyperconjugative effects of the N^6 methyl group.

Finally the difference in K_i values of inosine and guanosine (Table 3) also suggests that the electron density of N_1 is an important factor in binding to the active site. In both these nucleosides N_7 is the most basic nitrogen (29), but in guanosine the electron-donating 2-amino substituent will increase the electron density on N_1 enabling this nucleoside to bind more strongly to the enzyme.

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REFERENCES

1. A. N. Drury and A. Szent-Györgyi, *J. Physiol. (London)* **68**, 213 (1929).
2. H. N. Green and H. B. Stoner, "Biological Actions of the Adenine Nucleotides." Lewis, London, 1950.
3. R. M. Berne, *Physiol. Rev.* **44**, 1 (1964).
4. M. Rand, A. Stafford and R. H. Thorp, *J. Pharmacol. Exptl. Therap.* **114**, 119 (1955).
5. W. G. Naylor, *Australian J. Exptl. Biol. Med. Sci.* **37**, 109 (1959).
6. A. Angapindu, A. W. Stafford and R. H. Thorp, *Arch. Intern. Pharmacodyn.* **119**, 194 (1959).
7. D. A. Clarke, J. Davoll, F. S. Philips and G. B. Brown, *J. Pharmacol. Exptl. Therap.* **106**, 291 (1952).
8. R. H. Thorp and L. B. Cobbin, *Arch. Intern. Pharmacodyn.* **117**, 95 (1959).
9. H. Baer, G. I. Drummond and E. L. Duncan, *Mol. Pharmacol.* **2**, 67 (1966).
10. T. G. Brady and W. O'Connell, *Biochim. Biophys. Acta* **62**, 216 (1962).
11. H. J. Schaeffer, D. Vogel and R. Vince, *J. Med. Chem.* **8**, 502 (1965); H. J. Schaeffer and D. Vogel, *ibid.* **8**, 507 (1965).
12. J. G. Cory and R. J. Suhadolnik, *Biochemistry* **4**, 1729 (1965); *ibid.* **4**, 1733 (1965).
13. J. A. Johnson, H. J. Thomas and H. J. Schaeffer, *J. Am. Chem. Soc.* **80**, 700 (1958).

14. H. M. Kissman, C. Pidacks and B. R. Baker, *J. Am. Chem. Soc.* **77,** 18 (1955).
15. H. J. Schaeffer and H. J. Thomas. *J. Am. Chem. Soc.* **80,** 3738 (1958).
16. G. B. Brown and V. S. Weliky, *J. Org. Chem.* **23,** 125 (1958).
17. G. Gough and M. H. Maguire, *J. Med. Chem.* **8,** 866 (1965).
18. A. Stafford, T. R. Watson and M. J. Rand, *Biochim. Biophys. Acta* **18,** 318 (1955).
19. E. J. Conway, "Microdiffusion Analysis," p. 98. Crosby Lockwood, London, 1957.
20. H. M. Kalckar, *J. Biol. Chem.* **167,** 429, 461 (1947).
21. O. H. Lowry, N. J. Rosebrough, A. T. Farr and R. J. Randall, *J. Biol. Chem.* **193,** 265 (1951).
22. P. H. Everall and G. H. Wright, *J. Med. Lab. Tech.* **15,** 209 (1958).
23. A. Coddington, *Biochim. Biophys. Acta* **99,** 442 (1965).
24. G. N. Wilkinson, *Biochem. J.* **80,** 324 (1961).
25. M. Dixon and E. C. Webb, "Enzymes," 2nd ed., p. 325. Academic Press, New York, 1964.
26. C. S. Hanes, *Biochem. J.* **26,** 1406 (1932).
27. B. H. J. Hofstee, *Nature* **184,** 1296 (1959).
28. A. Albert and D. J. Brown, *J. Chem. Soc.* 2060 (1954).
29. J. J. Jones and R. K. Robins, *J. Am. Chem. Soc.* **85,** 193 (1963).