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CONVERSION OF 2,6-DIAMINO-9-(2-HYDROXYETHOXYMETHYL)PURINE TO ACYCLOVIR AS CATALYZED BY ADENOSINE DEAMINASE

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Abstract—Adenosine deaminase (ADA) was partially purified from several sources using affinity chromatography. These enzymes have the capacity to catalyze the deamination of 2,6-diamino-9-(2-hydroxyethoxymethyl)purine (A134U) to form the antiviral agent acyclovir [9-(2-hydroxyethoxymethyl)guanine]. Their relative substrate efficiencies (V_{max}/K_m) with A134U (standardized to adenosine = 100) were: dog ADA, 0.092; human ADA, 0.015–0.029; rat ADA, 0.025; calf ADA, 0.016; and *Escherichia coli* ADA, 0.0003. In addition to having the lowest efficiency with A134U, the bacterial ADA was also distinguished by its lack of binding of the mammalian ADA inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine and by its weak binding to the 9-(*p*-aminobenzyl)adenine-agarose affinity column. Four minor metabolites of A134U and acyclovir have been reported to be produced in the rat. These compounds are oxidized on either the C-8 position of the ring or the terminal carbon of the side chain. Neither acyclovir nor any of these metabolites produced significant inhibition of calf intestine ADA. The oxidized metabolites containing an N-6 amino group were extremely slow substrates of this enzyme.

2,6 - Diamino - 9 - (2 - hydroxyethoxymethyl)purine (A134U)† is currently being evaluated as an antiviral prodrug. A134U is metabolically transformed in the dog, rat and human to acyclovir [1, 2, ‡], a potent antiherpetic agent [3, 4]. Oral administration of this prodrug has an advantage over orally administered acyclovir because A134U is better absorbed from the gut and, therefore, produces higher plasma levels of acyclovir [2]. The hydrolytic deamination of A134U (see Fig. 1) is catalyzed by adenosine deaminase (ADA) [§, 5], an enzyme that is abundantly present in intestine and most other mammalian tissues (see Refs. 6 and 7 for data and reviews).

The purpose of the present study was to quantitate the kinetic parameters of the interaction of A134U with partially purified ADA from human, bovine, rat, canine and bacterial sources and to relate these values to the metabolic studies with A134U.

EXPERIMENTAL PROCEDURES

Materials. Calf intestinal ADA (sp. act. 200 I.U./mg at 25°) was purchased from Boerhinger Mannheim (Indianapolis, IN) and crystallized bovine serum albumin from Schwarz/Mann (Spring Valley, NY). A134U, [^{3}H]A134U (one tritium on each carbon of the ethoxy group), 8-hydroxyacyclovir, 2,6-diamino-8-hydroxy-9-(2-hydroxyethoxymethyl)purine, 9-carboxymethoxymethylguanine and 2,6-diamino-9-carboxymethoxymethylpurine were synthesized in these laboratories by L. Beauchamp, J. L. Kelley, and J. Scharver by procedures to be published elsewhere. Acyclovir [4] and EHNA [8] were prepared in the Wellcome Research Laboratories. [^{3}H]A134U was purified by high performance liquid chromatography (HPLC) on a Waters μBondapak C_{18} column in 2% ethanol. Solid human tissues were autopsy samples that had been stored at −80° for up to 2 months. Human erythrocytes and all other mammalian tissues were freshly all other

ADA assays. ADA was assayed spectrophotometrically at 265 nm ($\Delta E = -8.2\ mM^{-1}\ cm^{-1}$) at 37° in 100 mM potassium phosphate buffer, pH 6.9, and 0.5 mg bovine serum albumin/ml. The temperature was electronically maintained with a Gilford Thermoset. The standard assay contained 0.1 mM adenosine. One unit of ADA is that amount which catalyzes the formation of 1 μmole inosine/min under these conditions. Velocities were obtained from the initial linear rates recorded with a Gilford 250 spectrophotometer.

The conversion of [^{3}H]A134U to [^{3}H]acyclovir was assayed in the same buffer at 37°. The reactions were sampled at three time points by applying 10 μl to a cellulose thin-layer sheet prespotted with substrate and product carriers. Separation was achieved by ascending chromatography for 30 min in Tris : boric acid : H_2O (10.1 g : 1.93 g : 250 ml). [^{3}H]Acyclovir,

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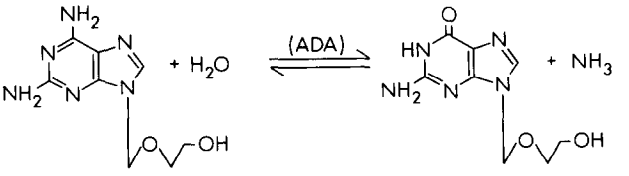
† Abbreviations: A134U, 2,6-diamino-9-(2-hydroxyethoxymethyl)purine; ADA, adenosine deaminase (EC 3.5.4.4); EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; PNPase, purine nucleoside phosphorylase (EC 2.4.2.1); and DTT, dithiothreitol.

‡ S. S. Good, H. C. Krasny and P. de Miranda, unpublished data.

§ Also observed by R. L. Miller of these laboratories in December 1973.



2,6-DIAMINO-
9-(2-HYDROXYETHOXYMETHYL)PURINE

ACYCLOVIR

Fig. 1. Hydrolytic deamination of A134U.

the u.v.-dark product spot ($R_f = 0.74$), and [^{3}H]A134U (u.v.-fluorescent spot at $R_f = 0.41$) were cut out, placed in 5 ml of Aquasol-2 (New England Nuclear Corp., Boston, MA) and quantitated by scintillation counting. Velocities were calculated from the linear slopes of a plot of product formation versus time. Less than 0.1% of the total radioactivity appeared in the product spot in mock reactions lacking ADA.

Kinetic constants. K_m and V_{max} values were determined by directly fitting the data to hyperbolas [9]. Most reported values are the average of duplicate determinations that agreed within 15%. The standard error on the K_m values ranged from 5 to 20%. I_{50} values for EHNA were calculated from rates that were obtained after preincubating EHNA and ADA for 5 min to establish equilibrium. The reactions were then initiated with a small volume of adenosine, and the very initial rate was measured before adenosine displaced any EHNA from its complex with ADA [10].

Protein assay. The refined [11] Coomassie Blue [12] assay was used to quantitate protein.

Purification of mammalian ADA. The affinity column described by Rossi *et al.* [13, 14] was used to obtain highly purified ADA. The gel contained 5.3 μmoles of bound 9-(*p*-aminobenzyl)adenine per ml of resin. Their procedures were generally followed except that the method for preparing extracts was simplified and the extracts were fractionated with ammonium sulfate before chromatography.

Human erythrocyte lysates were taken through the CM-Sephadex step described elsewhere [15]. The ADA was precipitated with 60% ammonium sulfate (36.1 g/100 ml at 4°), collected by centrifugation, resuspended in 50 mM potassium phosphate (pH 6.9) buffer with 1 mM DTT (Buffer A), and dialyzed overnight against the same buffer. The chromatography is described below.

The solid mammalian tissues were cut into small pieces, washed extensively with cold isotonic saline, blotted, and weighed. Saline, 0.5 ml/g tissue, was then added and the tissues were disrupted in a Polytron tissue homogenizer. The supernatants obtained from a 45,000 *g* centrifugation were fractionated at 4° with ammonium sulfate. The 40–95% fraction was collected and dialyzed as described above for all samples except the human jejunum. Since the ADA from this source was divided between the 0–40% and 40–95% fractions, these fractions were combined.

The dialyzed samples of mammalian tissues were pumped at 0.25 ml/min onto the affinity column (approximately 3 ml bed vol. per 20 g wet wt of starting tissue) at room temperature. The extraneous protein was washed through with 100 mM potassium phosphate, pH 6.9, buffer containing 1 mM DTT. The ADA was then eluted in a sharp peak with 100 mM potassium phosphate buffer, pH 7.8, with 1 mM DTT and 4 mM guanylurea as previously described [11, 12]. The solutions of enzyme were brought to 0.5 mg bovine serum albumin/ml, exhaustively dialyzed against Buffer A, and stored at −80°.

Purification of ADA from Escherichia coli. The mutant SØ540 [16], which is lacking the *deo operon* and consequently is deficient in purine nucleoside phosphorylase,* was grown in Vogel & Bonner salts [19] with 60 g/l of Difco yeast extract and 20 mg/l of thiamine. The collected cells were washed with cold buffer A, pelleted, resuspended in 2 vol. of cold water, and sonicated. The supernatant fraction was obtained by centrifugation and brought to 0.5% protamine sulfate by the addition of a 2.5% solution of protamine sulfate. The subsequent supernatant was fractionated with ammonium sulfate at 4°. The 35–85% fraction was collected, dialyzed against Buffer A, and loaded onto the affinity column as described above. Although this ADA was only retarded and not steadfastly retained, it was separated from the bulk of the protein. Further purification was achieved by reconcentration and rechromatography. The ADA was finally concentrated by precipitation with 80% ammonium sulfate, dialyzed against 200 mM Buffer A with 20% ethylene glycol and 0.1 mM EDTA [20], and stored at 4°.

Reuse of the affinity column. After chromatography, the column was washed with a solution of 6 M urea and 1 M KCl and then reequilibrated in Buffer A and stored at 4°. Affinity resin treated in this manner could be reused many times.

RESULTS

Enzymatic conversion of A134U to acyclovir. Initial studies were performed with purified calf intestine ADA. The complete hydrolytic deamination of A134U to form acyclovir (Fig. 1) was monitored both spectrophotometrically and radiochemically. The spectral traces of Fig. 2 show isosbestic points at 233 and 273.5 nm and a spectral change at

* Although mammalian purine nucleoside phosphorylase does not efficiently cleave adenosine [17], the bacterial enzyme does [18] and therefore was avoided.

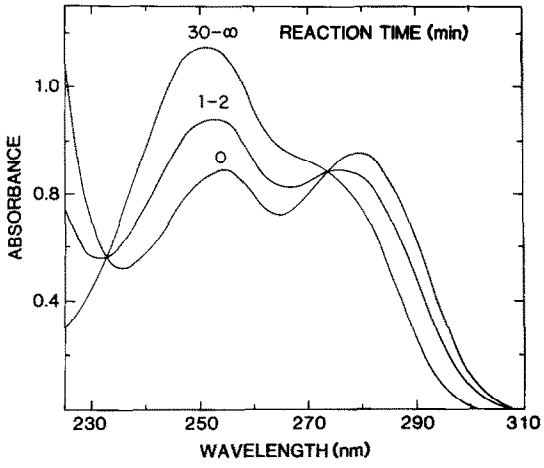


Fig. 2. Spectral evidence for the conversion of A134U to acyclovir. At T = 0 min, the spectrum of a 0.1 mM solution of A134U in 100 mM potassium phosphate buffer, pH 6.9, was recorded with buffer in the reference cuvette at 37°. Calf intestinal ADA (10 μl) was then added to the sample and reference cuvettes, and the spectra were recorded at the indicated times. A Beckman Acta MVI spectrophotometer was used.

250 nm with an extinction coefficient of 4.8 mM^{-1} cm^{-1}. The final spectrum was identical to that of acyclovir. The reaction could be inhibited completely by EHNA, or deoxycoformycin, both of which are highly specific inhibitors of ADA [8, 10, 21]. The radiochemical assay proceeded until more than 99% of the radioactivity co-migrated with acyclovir (see Experimental Procedures).

Comparative studies with ADA from several sources. ADA was purified from three human tissues, rat intestine, dog intestine and *E. coli* as described above. The specific activity of ADA was 8.4 units/mg for human erythrocytes, 5.1 units/mg for the enzyme from *E. coli* and ⩾60 for ADA from the other tissues. Higher specific activities were sometimes obtained by extending the buffer wash of the column-bound ADA and by rechromatography.

The addition of bovine serum albumin greatly stabilized the enzymes for the reactions at 37° but was less effective in stabilizing them to long-term storage at −80°. A slow, but steady loss in activity of mammalian ADA occurred over several weeks. The bacterial enzyme lost only 20% of its activity over 10 weeks at 4°.

Contamination by purine nucleoside phosphorylase (PNPase) was assessed in the preparations of ADA from human erythrocytes, human spleen, rat intestine, dog intestine, and *E. coli*. All preparations except the erythrocytes were free (less than 0.05% of the adenosine-deaminating activity) of PNPase. The latter preparation had a 2-fold excess of PNPase. Fortunately, this enzyme did not interfere with the present assays. This is because adenosine has negligible substrate activity with mammalian PNPase [17]. Also, although inosine is an efficient substrate, it has only a negligible spectral change at 265 nm ($\Delta E = -0.6\ mM^{-1}$, cm^{-1}) for its cleavage to hypoxanthine. Furthermore, A134U and acyclovir are not substrates for PNPase.* Since bacterial PNPase catalyzes the rapid cleavage of adenosine [18], it was imperative to remove the detectable traces of this activity from the preparation of ADA from *E. coli*. The affinity column accomplished this.

The K_m and V_{max} values of A134U with these deaminases are presented in Table 1. To further characterize the enzymes for comparative purposes, the K_m and V_{max} values for adenosine and the I_{50} values for EHNA are also presented.

EHNA has been shown to bind slowly to ADA from calf intestine and human erythrocytes [10]. This slowness was detected by a 2-min period of rate deceleration when reactions with adenosine (EHNA present) were initiated with ADA. Furthermore, if EHNA and ADA were preincubated until the binding had reached equilibrium, the displacement of EHNA by adenosine was also slow. In this case, an acceleration of the rate was observed. In the present study, EHNA demonstrated a similar slow-binding to ADA from human, bovine and canine sources. The I_{50} values reported here were determined from rates that were measured at the time when the inhibition was maximal (see Experimental Procedures). This, however, was not the case with rat intestinal ADA. At 37°, the initial velocities were always constant. Therefore, the binding of EHNA and this enzyme appeared to establish equilibrium rapidly and, following the addition of adenosine, the reaction appeared to reach steady state rapidly. Slower binding and slower displacement of EHNA could be detected when the temperature was lowered to 20°.

Interactions of the trace metabolites of A134U with ADA. Rats dosed with A134U produced trace amounts of oxidized metabolites of A134U and acyclovir [1]. These metabolites were tested as inhibitors and/or substrates of calf intestinal ADA. Neither acyclovir nor the oxidized metabolites, 8-hydroxyacyclovir, 9-carboxymethoxy-methylguanine, 2,6-diamino-8-hydroxy-9-(2-hydroxyethoxymethyl)purine, and 2,6-diamino-9-carboxymethoxymethylpurine, produced any inhibition when assayed at 80 μM with adenosine at 40 μM. Preincubation of these compounds with ADA did not alter these results. Therefore, if they are inhibitors, their K_i values would have to be very large.

The calf intestine ADA was capable of catalyzing the extremely slow deamination of 2,6-diamino-9-carboxymethoxymethylpurine to form 9-carboxymethoxymethylguanine. At 0.1 mM, the deamination proceeded at 5×10^{-4}% of the rate observed with adenosine. The product was identified by its spectral properties. This deamination was inhibited completely by deoxycoformycin. The deamination of 2,6-diamino-8-hydroxy-9-(2-hydroxyethoxymethyl)purine appeared to occur at 3×10^{-5}% of the adenosine rate and was also inhibited by deoxycoformycin.

Additional studies with E. coli *ADA.* Since some of the properties of the bacterial ADA were ostensibly different than those of the other ADAs, a few

* J. V. Tuttle and T. A. Krenitsky, personal communication.

Table 1. Kinetic constants for various adenosine deaminases

Source	Adenosine		A134U		EHNA
	K_m (μM)	V_{max} (μmoles/min/unit)	K_m (μM)	V_{max} (μmoles/min/unit)	I_{50} (nM)
Dog jejunum	59	1.60	2,600	0.064	17
Human erythrocytes	34	1.34	700	0.0082	11
Human spleen	26	1.25	530	0.0069	12
Human jejunum	26	1.26	600	0.0044	8
Rat intestine	21	1.21	850	0.012	45 [K_i = 8 nM]*
Calf intestine	32	1.32	500	0.0032	10
E. coli (PNPase$^-$)	61	1.54	6,000	0.0004	$\geqslant$27,000

* See Discussion.

experiments were performed to further characterize this enzyme. The deamination of adenosine was verified with a reaction containing [^{14}C]adenosine. More than 95% of the total radioactivity cochromatographed with inosine, $R_f = 0.93$ (R_f adenosine = 0.70) after thin-layer chromatography as described in Experimental Procedures. Deoxycoformycin was tested as an inhibitor. It produced slow-binding inhibition with an I_{50} of 0.3 nM determined at the time of maximal inhibition (see above). Coformycin was also reported to be an inhibitor with a K_i of 30 nM [20]. Furthermore, tubercidin, which is devoid of substrate activity with human erythrocyte ADA [22], was also not a substrate for this enzyme.

DISCUSSION

ADA from a variety of mammalian sources clearly has the capacity to catalyze the deamination of A134U and thereby produce acyclovir. Earlier studies have demonstrated that mammalian ADA catalyzes the conversion of diaminopurine riboside [22, 23], deoxyriboside* and arabinoside [24] to their corresponding guanine nucleosides. These glycosidic nucleosides were considerably more efficient substrates (V_{max}/K_m) than the present acyclic compound which lacks the 2′, 3′-carbon bridge of the sugar. A similar relationship was noted between adenosine and 9-(2-hydroxymethoxymethyl)adenine, the first acyclic nucleoside analog to be reported as a substrate of ADA [25].

The low substrate efficiency of A134U is probably responsible for its incomplete metabolism to acyclovir in the rat where about 30% of the dose recovered in the urine was not deaminated [1]. Of all the enzymes tested, the substrate efficiency of A134U was highest with canine ADA, This is consistent with the recent finding that >80% of the dose recovered in the urine of dogs treated with A134U was acyclovir.† The extent of the deamination in humans [2] is more similar to that in rats than in dogs and correspondingly correlates with the relative substrate efficiency of A134U with human ADA. However, it is obvious that since the substrate efficiencies are so low, the patient's level of ADA may also play a crucial role in the amount of acyclovir that is formed. The present data further indicate that it is unlikely that acyclovir or any of the trace amounts of the oxidized metabolites [1, 2] of acyclovir or A134U would have any significant pharmacological interactions with ADA.

ADA from *E. coli* was investigated as a possible explanation for the finding that >97% of the dose recovered in rat feces was deaminated [1]. Unexpectedly, the kinetic constants with this enzyme and A134U were rather unfavorable. However, it is possible that protracted exposure of high lumenal concentrations of A134U to large quantities of bacterial ADA could result in deamination. It is also possible that the efficiency of ADA from *E. coli* is not representative of the efficiency of ADA from the various other intestinal bacteria.

The *E. coli* ADA also differs from mammalian ADA with respect to the nature of the hydrophobicity of the area adjacent to its catalytic site. This region has been well defined in calf intestine ADA [8, 26] and is largely responsible for the snugness of the binding of EHNA, the inhibitor, and 9-(*p*-aminobenzyl)adenine, the ligand of the affinity column used in these studies. The differences are manifest by a diminished ability of bacterial ADA to bind these compounds. ADA from *E. coli* B-96‡ and the 100,000 molecular weight ADA_2 (see Ref. 27 for review) have also been shown to be resistant to inhibition by EHNA.

Since the *E. coli* ADA was very sensitive to the transition-state analog inhibitors (coformycin and deoxycoformycin) of mammalian ADA [10, 28], it probably functions with a similar reaction mechanism. The sensitivities of AMP deaminase [29], adenine deaminase [30] and these adenosine deaminases to coformycin and deoxycoformycin indicate that they all probably produce a common tetrahedral intermediate [31] on the adenine nucleus which is closely mimicked by the heterocyclic moiety of these analogs. Another similarity between mammalian [22] and *E. coli* ADA is that neither was capable of catalyzing the deamination of tubercidin (7-deazaadenosine).

With the exception of rat ADA, the ADA from

* T. Spector, unpublished observation.

† S. S. Good, H. C. Krasny and P. de Miranda, unpublished data.

‡ The inability of EHNA to inhibit bacterial ADA was observed originally in these laboratories by T. A. Krenitsky and L. Pressel (unpublished data).

all the mammalian sources was inhibited by EHNA with an I_{50} of about 10 nM. Under the special conditions in which these I_{50} values were determined (see text), they approximate the K_i [10]. In this case, this K_i is an overall inhibition constant that includes an intitial enzyme–inhibitor complex and a tighter complex that slowly forms from the initial complex [32]. Because, at 37°, the tightly formed rat ADA–EHNA complex was in rapid equilibrium with the initial complex, steady state was immediately reached and only a steady-state I_{50} could be determined. The conventional conversion [33] of this value into a K_i produced a value of 8 nM which is similar to the K_i determined for ADA from the other mammalian sources.

In conclusion, mammalian ADA catalyzed the conversion of the prodrug, A134U, to the antiviral agent, acyclovir. Although the reaction was characterized by a high K_m and a low V_{max}, the combination of high levels of ADA and low levels of the competing natural substrate [34] is undoubtedly responsible for the conversion *in vivo* [1, 2, *].

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* S. S. Good, H. C. Krasny and P. de Miranda, unpublished data.