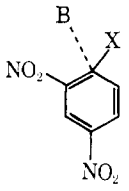
ient formation of a tetrahedral intermediate involving enzyme (or enzyme-bound water) and substrate (II).



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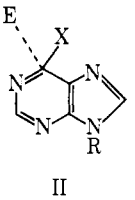

II

In the case of calf intestinal adenosine deaminase, where there is variation of V_{max} with leaving group, it appears likely that stretching of the bond to leaving group may occur in the inversion in order of mobility of ammonia and chloride as between 6-substituted purine ribonucleosides and 6-substituted 2-aminopurine ribonucleosides (Table III) illustrates the subtle balance between bond making and bond breaking which may apply in this case. However there are substrates of the mammalian enzyme for which V_{max} values are very much lower than that for adenosine (Chassy and Suhadolnik, 1967; Baer *et al.*, 1968) and it apears likely that bond breaking dominates the kinetic behavior of these substrates.

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Ring-Modified Substrates of Adenosine Deaminases*

R. Wolfenden, J. Kaufman, and J. B. Macon

ABSTRACT: Adenosine deaminases (adenosine aminohydrolase, EC 3.5.4.4) from the mould *Aspergillus oryzae* and from calf intestine catalyze deamination of 4-aminopteridine, 1-methyladenosine, 3-β-(D-ribofuranosyl)adenine, and unsubstituted adenine. Unsubstituted pteridine is an effective inhibitor of both enzymes.

When adenine derivatives are compared, electron-withdrawing groups are found to increase the limiting rate of enzymatic deamination. The presence of ribose as a substituent on either the 3 or the 9 position, or the replacement of the 8-carbon by sulfur or oxygen, result in large increases in V_{max} as compared with adenine. Similarly, 8-trifluoromethylpurine is a much more effective inhibitor than unsubstituted purine.

Adenosine deaminases (adenosine aminohydrolase, EC 3.5.4.4) have been isolated as pure proteins from calf intestine (Brady and O'Connell, 1962) and from *Aspergillus oryzae* (takadiastase) (Wolfenden *et al.*, 1968). Both enzymes appear to be protein catalysts not requiring cofactors. Both exhibit considerable nonspecificity. The enzyme from takadiastase rapidly deaminates adenine nucleosides, nucleotides, and oligonucleotides (Wolfenden *et al.*, 1967). Both enzymes catalyze hydrolytic cleavage of nitrogen-, halogen-, oxygen-, and sulfur-leaving groups from 6-substituted purine ribonucleosides (Cory and Suhadolnik, 1965; Wolfenden, 1966; Baer and Drummond, 1966; Wolfenden and Kirsch, 1968).

This paper explores another aspect of the catalytic versatility

* From the Program in Biochemical Sciences, Frick Chemical Laboratory, Princeton University, Princeton, New Jersey 08540. *Received January 22, 1969.* Supported by Research Grant No. GM-12725 from the National Institutes of Health, U. S. Public Health Service.

TABLE I: Substrates for Adenosine Deaminases.

	Wavelength (mμ)	$\Delta\epsilon_M$ (deamination)	Ref
Adenosine (I)	260	7.8×10^3	*a*
3-(β-D-Ribofuranosyl)-adenine (II)	285	7.9×10^3	*b*
1-Methyladenosine (III)	260	8.2×10^3	*c*
Adenine (IV)	260	5.4×10^3	*a*
4-Aminopteridine (V)	350	3.9×10^3	*d*
7-Amino[1,2,5]oxydiazolo[3,4-*d*]pyrimidine (VI)	340	6.1×10^3	
7-Amino[1,2,5]thiadiazolo[3,4-*d*]pyrimidine (VII)	340	7.8×10^3	*e*

[a] Beaven *et al.* (1955). [b] Wolfenden *et al.* (1966). [c] Macon and Wolfenden (1968). [d] Mason (1954). [e] Shealy *et al.* (1962).

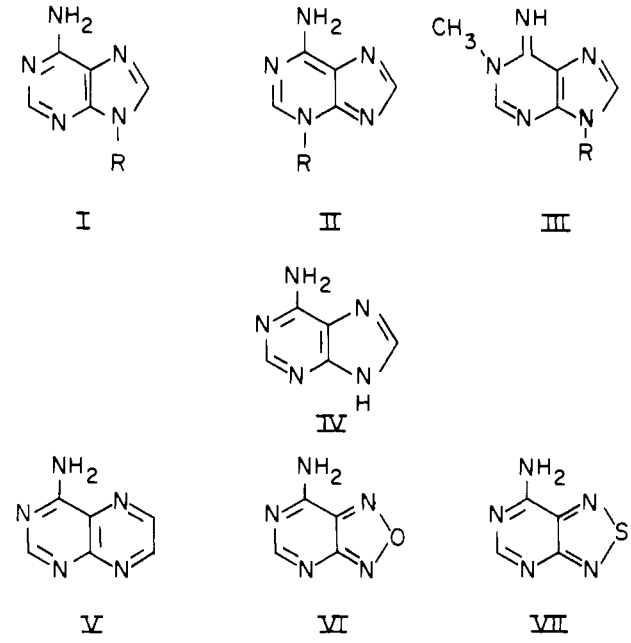

FIGURE 1: Ring-modified substrates of adenosine deaminase.

of these enzymes, the deamination of adenosine analogs in which the heterocyclic ring system has been drastically altered. Despite the resulting redistribution of electrons, these compounds continue to act as substrates, a fact which must be explained in any attempt to formulate a general mechanism. Of particular interest are 1-methyladenosine, 3-(β-D-ribofuranosyl)adenine, 4-aminopteridine, and several compounds related to adenine, most notably its 8-oxygen and 8-sulfur analogs. A preliminary report of the deamination of isoadenosine has appeared (Wolfenden *et al.*, 1966).

Experimental Procedures

Pure preparations of adenosine deaminase from takadiastase and from calf duodenum were obtained as described previously (Wolfenden *et al.*, 1968). 1-Methyladenosine was prepared from the hydriodide by the method of Jones and Robins (1963). 3-(β-D-Ribofuranosyl)adenine was the generous gift of Dr. Nelson J. Leonard. 7-Amino[1,2,5]oxydiazolo-[3,4-*d*]pyrimidine and 7-amino[1,2,5]thiadiazolo[3,4-*d*]pyrimidine prepared by the method of Shealy *et al.* (1962) were the generous gift of Mr. Ben Evans. Unsubstituted pteridine was prepared by the method of Albert (1951). Purine, purine ribonucleoside, and 4-aminopteridine were obtained from Cyclo Chemical Co. and used without further purification. 8-Trifluoromethylpurine was prepared by condensation of 4,5-diaminopyrimidine with trifluoroacetic acid and isolated by sublimation (Bendich *et al.*, 1957; Albert, 1966).

All substrates, when incubated overnight with high concentrations of enzymes (0.1 mg/ml) in potassium phosphate buffer (0.1 M, pH 6.50) at 25°, were completely converted into the corresponding hydroxy compounds. Kinetic parameters were obtained from reciprocal plots of the dependence of deamination rate upon substrate concentration. V_{max} values were recalculated in terms of concentration using changes in molar extinction coefficient at the wavelength at which kinetic measurements were made (Table I). Kinetic measurements were made at 25° in potassium phosphate buffer (0.1 M, pH 6.50) with a Zeiss PMQ II spectrophotometer, using cuvets of 1-cm or 1-mm path lengths. In the case of 1-methyladenosine, measurements were also made in Tris-Cl buffer (0.1 M, pH 8.9).

Results

Initial rates of deamination of substrates by adenosine deaminases followed normal Michaelis–Menten kinetics. K_m and V_{max} values, obtained from double-reciprocal plots and corrected for the relative change in molar extinction coefficients upon deamination (Table I), are shown in Table II. These values apply in 0.1 M potassium phosphate buffer (pH 6.50) at 25°. In the case of 1-methyladenosine, measurements were also made in 0.1 M Tris-Cl buffer, as indicated. Kinetic parameters for 1-methyladenosine showed little variation between pH 6 and 9 with either enzyme.

Purine, 8-trifluoromethylpurine, purine ribonucleoside, and unsubstituted pteridine were found to be competitive inhibitors of adenosine deamination. Table III shows inhibition constants obtained for these compounds in 0.1 M potassium phosphate buffer (pH 6.50) at 25°.

Discussion

The ability of adenosine deaminases to hydrolyze ammonia from such a wide variety of compounds presumably reflects the presence of an electron-deficient carbon atom at the center of substitution in all substrates. Deamination of this array of substrates suggests that: (a) there seem to be few if any absolute restrictions on ring positions at which substitutions may be made; (b) the distribution of double bonds in the most stable canonical forms (Figure 1) may vary in almost every conceivable fashion; and (c) expansion of the imidazole ring does not abolish activity.

In view of the fact that adenosine appears to be found by takadiastase adenosine deaminase only in its neutral form

TABLE II: Relative Rate Constants for Deamination of Adenine Analogs by Adenosine Deaminases at 25° in 0.1 M Potassium Phosphate (pH 6.50).[a]

	Takadiastase		Calf Duodenum	
	V_{max} (rel)	K_m (M × 10^4)	V_{max} (rel)	K_m (M × 10^{-5})
Adenosine (I)	100	2.4	100	3.1
3-(β-D-Ribofuranosyl)adenine (II)	5.1	50	0.52	7.7
1-Methyladenosine (III), pH 6.50	0.39	23.2	0.17	9.6
1-Methyladenosine, pH 8.90	0.30	19	0.18	10.1
Adenine (IV)	0.23	7.2	0.0014	15
4-Aminopteridine (V)	0.88	36	0.58	10.5
7-Amino[1,2,5]oxydiazolo-[3,4-*d*]pyrimidine (VI)	1.01	41.6	0.79	38
7-Amino[1,2,5]thiadiazolo-[3,4-*d*]pyrimidine (VII)	12.5	17.5	7.4	333
2′-Deoxyadenosine			93[b]	2.2[b]
2′-Deoxy-8-azaadenosine			460[b]	12.5[b]

[a] Except as indicated. [b] Frederiksen (1966).

(Wolfenden *et al.*, 1967), it is somewhat surprising that 1-methyladenosine, the pK_a of whose conjugate acid is 8.2 (Macon and Wolfenden, 1968), is almost equally effective as a substrate at pH 6.5 and 9 (Table II). It appears that this compound acts as a substrate in both its neutral or conjugate acid forms, although deamination of either form is rather slow.

A conceivable mechanism for enzymatic deamination of adenosine would be by enzyme stabilization of the rare imino tautomer, as contrasted with the 6-amino tautomer of adenosine which predominates by a factor of more than 10^4 (Wolfenden, 1969a). The imino tautomer might then be subject to rapid hydrolysis. However these enzymes are active on substrates with leaving groups other than ammonia, so that this is unlikely to be an essential feature of the catalytic mechanism. Enzyme activity on 1-methyladenosine (whose neutral species is already in the 6-imino form) and on 9-(β-D-ribofuranosyl)-adenine (Wolfenden *et al.*, 1966) are inconsistent with imine formation as a sufficient explanation for catalysis.

An alternative mechanism, consistent with the available kinetic evidence (Wolfenden, 1969b), involves rate-limiting attack on the position of purine derivatives to form a tetrahedral intermediate of the kind generally encountered in nucleophilic aromatic substitution. This mechanism can be reconciled with the ability of the present ring-modified compounds to act as substrates, since all can form tetrahedral intermediates of similar structure (Figure 2), where E may be enzyme or water.

If nucleophilic attack constitutes a part of the rate-limiting step in the reaction, V_{max} values might be expected to be raised by the presence of electron-withdrawing groups in the ring system. It is therefore interesting that V_{max} for adenine is enhanced by the substitution of oxygen and sulfur atoms for the 8-carbon, V_{max} for deoxyadenosine is en-

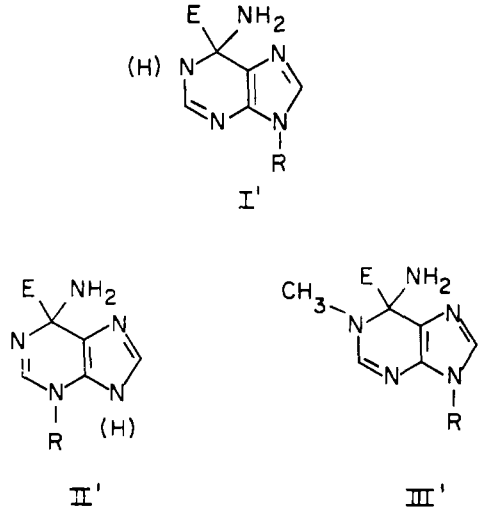

FIGURE 2: Possible tetrahedral intermediates in heteroaromatic substitution.

TABLE III: Constants for Competitive Inhibition of Adenosine Deaminases at 25° in 0.1 M Potassium Phosphate Buffer (pH 6.50).

	K_i (Takadiastase), M × 10^{-5}	K_i (Calf), M × 10^{-5}
Purine ribonucleoside	3.7	0.93
Purine	260	
8-Trifluoromethylpurine	17	
Pteridine	2.3	12

hanced by substitution of nitrogen for the 8-carbon (Frederiksen, 1966), and substitutions of ribose at the 3 or 9 positions of adenine result in very large rate enhancements (Table II). There is no basis for excluding the possibility that "induced fit" (Koshland, 1959) may play a role in the ribose effect; however, it appears that sulfur at the 8 position can also largely overcome the deficiencies of adenine as a substrate insofar as V_{max} is concerned.

The observed effects of structure on inhibition constants suggest that electron deficiency in the neighborhood of the 6 position may contribute to tight binding. Thus purine becomes a much more effective inhibitor when ribose is present at the 9 position or when trifluoromethyl is present at the 8 position (Table III). Strong inhibition by pteridine as compared with purine is also consistent with this interpretation, since pteridine exceeds purine in its tendency to form covalent hydrates, an indication of electrophilicity (Albert, 1967).

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