Catalysis of the Covalent Hydration of Pteridine by Adenosine Aminohydrolase†

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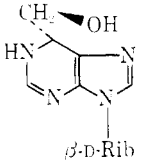
ABSTRACT: Adenosine deaminase catalyzes the stereospecific hydration of pteridine and dehydration of pteridine hydrate with an efficiency comparable to that observed for deamination of 4-aminopteridine. Kinetic similarities between the hydration and deamination reactions suggest that, in deamination, water attacks the substrate directly, and that the transition state in this reaction is reached before a tetrahedral intermediate is fully developed.

Adenosine deaminase from mammals and microorganisms catalyzes the hydrolytic removal of various leaving groups from several heterocyclic ring systems. Chemical precedent suggests that these reactions proceed by nucleophilic aromatic substitution, but does not help to distinguish between two possibilities. Water may attack the substrate directly, or may instead react with a covalent purinyl-enzyme intermediate analogous to the acyl-enzymes formed by many hydrolases (Wolfenden, 1969).

Adenosine deaminase has been found to be strongly inhibited by an analog (1) which bears a suggestive resemblance to a hypothetical tetrahedral intermediate (**2**) in *direct water attack* on adenosine (Evans and Wolfenden, 1970). In this report, portions of which have appeared in a preliminary communication (Evans and Wolfenden, 1972), we wish to describe an analog, not of the proposed intermediate, but of the partial reaction which leads to its formation.



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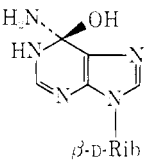

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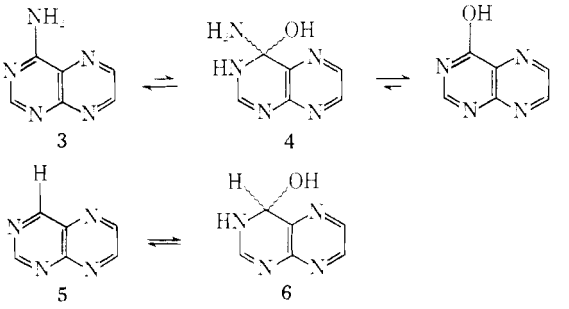
Adenosine deaminase catalyzes the deamination of 4-aminopteridine (**3**) (Scheme I). It is also inhibited by *unsubstituted* pteridine **5** (Wolfenden *et al.*, 1969). The meaning of this latter observation is clouded by the known ability of pteridine to undergo spontaneous hydration in neutral aqueous solution, yielding an equilibrium mixture containing 22.5% of the 3,4-monohydrate **6** (Perrin, 1962). Solutions of essentially pure anhydrous pteridine or pure pteridine hydrate are readily prepared, and it should therefore be possible to determine separately the properties of pteridine and its covalent hydrate as enzyme inhibitors. When this was attempted, variable results were obtained depending on the order of addition of reagents to the assay mixture. Further examination showed that pteridine reacts with adenosine deaminase not as a passively bound inhibitor, but as a *substrate*. Adenosine deaminase serves as a remarkably efficient and *stereospecific* catalyst of the covalent hydration of pteridine.

SCHEME I



Experimental Section

Pteridine was prepared as described by Albert *et al.* (1951). Calf duodenal adenosine deaminase was obtained from Boehringer-Mannheim Corp. (as a suspension of *ca.* 10 mg/ml in 2.8 M ammonium sulfate). 6-Hydroxymethyl-5,6-dihydropurine ribonucleoside (**1**) was synthesized according to Evans and Wolfenden (1970). 4-Hydroxypteridine (4-(3*H*)pteridinone) was prepared by the method of Albert *et al.* (1951).

Pteridine was converted to the hydrate **6** by acidifying aqueous pteridine (5–50 μl, ~2×10^{-2} M) with an equal volume of 0.04 N HCl, and allowing this mixture to stand for *ca.* 3 sec (Perrin, 1962). Upon quenching in neutral buffer (10 ml of 0.1 M potassium phosphate, pH 7.5) the resulting solution of hydrate reverted slowly to the equilibrium mixture. Initial velocities of this reaction were obtained at 25° by following the decrease in absorbance of a 3-ml sample of the mixture at 318 nm (Table I) using a Zeiss PMQ II spectrophotometer (1-cm cuvet).

After a short interval to allow precise determination of the nonenzymatic reaction rate, 1 μl of enzyme suspension was added with mixing and the reaction rate again was determined. Less than 5% conversion to product occurred in the time required to ascertain both enzymatic and nonenzymatic reaction rates, and in no case did the nonenzymatic reaction contribute more than 15% of the uncorrected rate observed in the presence of enzyme.

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The reverse (hydration) reaction was studied by adding a measured aliquot (50 μl or less) of anhydrous pteridine in dimethylformamide ($\sim 2 \times 10^{-2}$ M) to 10 ml of 0.1 M potassium phosphate buffer (pH 7.5). The nonenzymatic and enzymatic reaction rates were determined by the increase in absorbance at 318 nm. Control experiments showed no detectable inhibition by dimethylformamide at this concentration level.

The observed half-time (in 0.1 M potassium phosphate buffer, pH 7.5, 25°) for nonenzymatic reequilibration of pure pteridine or pteridine hydrate ($t_{1/2}$ = 22 min) was long enough to permit the spectrum of each to be scanned before appreciable reequilibration had occurred. Spectra of pteridine and its hydrate, obtained on a Coleman/Perkin-Elmer Model 124 uv–visible spectrophotometer (scan time 150 sec, $\lesssim 8\%$ reaction), are illustrated in Figure 1 along with the spectrum of the equilibrium mixture. These curves are in reasonable agreement with the point-by-point spectra reported by Perrin (1962).

To obtain the pH profile shown in Figure 2, dehydration of pteridine hydrate was examined in the following buffers (all 0.1 M): pH 5.5, acetic acid–KOH; pH 6.0, 6.5, 7.0, and 7.5, KH_2PO_4–K_2HPO_4; pH 8.0 and 8.5, Tris–HCl; pH 9.0 and 9.5, glycine–KOH. In each case, the ionic strength was maintained at 1.0 by addition of KCl.

Deuterium isotope effects were evaluated in aqueous potassium phosphate buffer (1 M, pH 7.5) which was alternatively diluted 10-fold in H_2O and D_2O. Initial rates of hydration and dehydration in each buffer were determined in triplicate (total pteridine concentration = 1.06×10^{-4} M) in the presence and absence of enzyme.

Optical rotation experiments were conducted in a Cary Model 60 recording spectropolarimeter (1-cm cuvet) measuring optical rotation as a function of time (0.1° full scale) at fixed wavelength (365 nm).

Results

In agreement with the results of Inoue and Perrin (1963), the nonenzymatic dehydration of pteridine hydrate over the pH range 5.5–9.5 was found to be subject to general and specific acid and base catalysis with a rate minimum at pH 7.5 ± 0.1. The pH dependence of the observed rates, extrapolated to zero buffer concentration at constant ionic strength (1.0), is illustrated in Figure 2 (compare Figure 3 in Inoue and Perrin, 1963). pH was maintained at 7.5 in subsequent kinetic studies in order to maximize the difference between enzymatic and nonenzymatic reaction rates. At this pH, in 0.1 M potassium phosphate buffer, the observed half-time for dehydration was 22 min at 25°. The reverse (hydration) reaction, under identical conditions of pH, concentration, etc., exhibited the same net half-time, 22 min.

The initial rate of the enzyme-catalyzed dehydration, corrected by subtraction of the nonenzymatic rate, was followed (pH 7.5) at a variety of pteridine concentrations, yielding a linear double-reciprocal plot (Figure 3). V_{max} and K_m were obtained by a computer fit (Hanson *et al.*, 1967) of the experimental data to the Michaelis–Menten equation. The reverse reaction, pteridine hydration, was examined in similar fashion; the double-reciprocal plot appears in Figure 4. The computer-calculated kinetic parameters for both reactions are listed in Table I. Similar experiments were carried out with a crude preparation of Takadiastase adenosine deaminase (Wolfenden *et al.*, 1968), and the results are also presented in Table I.

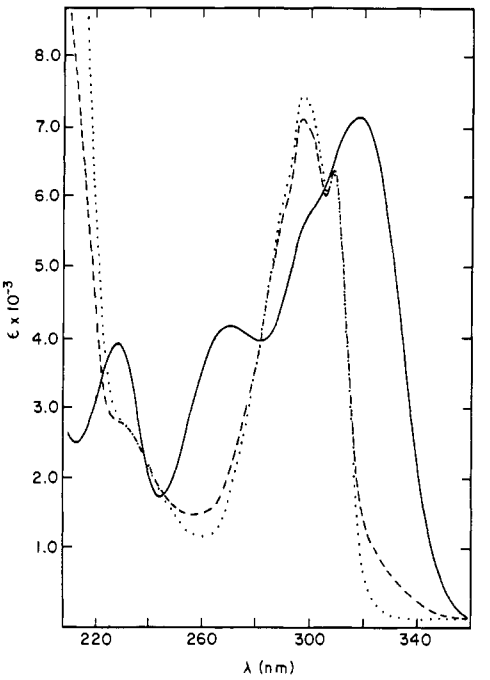


FIGURE 1: Ultraviolet absorbance spectra of pteridine (....), pteridine hydrate (——), and their equilibrium mixture (– – – –) in 0.1 M potassium phosphate buffer, pH 7.5, at 25°.

Pteridine and its hydrate, generated as described above, were also examined as inhibitors of the action of adenosine deaminase on adenosine. Under the conditions employed, the rate of the pteridine–hydrate equilibration was sufficiently slow to permit the assays to be carried out with nearly static concentrations of pteridine or hydrate. Determination of adenosine deamination rate in the presence of a variety of substrate and inhibitor concentrations and construction of the double-reciprocal plots (Figures 5 and 6) allowed evaluation of K_i for both pteridine and its hydrate (see Table I). These were in close agreement with the corresponding K_m values, suggesting that the latter were *true binding constants*.

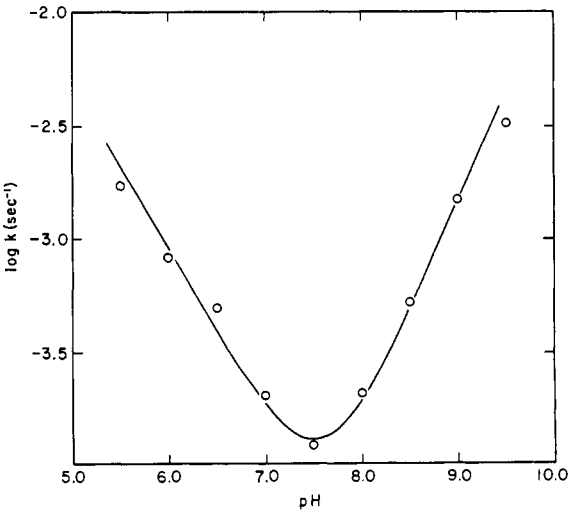


FIGURE 2: pH dependence of the rate of dehydration of pteridine hydrate in aqueous buffers at ionic strength 1.0. Function plotted is the logarithm of the first-order rate constant (sec^{-1}) *vs.* pH.

TABLE I: Kinetic Parameters for Adenosine and Selected Pteridines as Substrates–Inhibitors of Adenosine Deaminases (0.1 M phosphate buffer, pH 7.5, 25°).

Enzyme Source	Substrate (Reaction)				
	Adenosine (Deamination)	4-Aminopteridine (Deamination)	Pteridine (Hydration)	Pteridine Hydrate (Dehydration)	4-Hydroxy-pteridine
Mammalian					
K_m (M), $\times 10^5$	3.1[a]	10.5[a]	16.3 ± 0.2[e]	3.55 ± 0.43[b,e]	
K_i (M), $\times 10^5$			10 ± 4	2.7 ± 1[b]	66 ± 8
V_{max} (μmol/min per mg of enzyme)	217[c]	1.26[a]	3.56 ± 0.14[e]	5.00 ± 0.15[c]	
V_{max}/K_m, $\times 10^{-5}$	70.0	0.12	0.22	1.40	
Fungal					
K_m (M), $\times 10^4$	2.4[a]	36.0[a]	1.7 ± 0.5	0.95 ± 0.2[b]	
V_{max} (μmol/min per mg of enzyme)	517[c]	4.56[a]	0.073 ± 0.01	0.38 ± 0.07	
V_{max}/K_m, $\times 10^{-4}$	205	0.13	0.043	0.40	
Wavelength, nm ($\Delta\epsilon \times 10^3$)[d]	260 (−7.8)[a]	350 (−3.9)[a]	318 (+7.4)	318 (−7.4)	

[a] Wolfenden *et al.* (1969). [b] Assumes one enantiomer only is bound, see text. [c] Wolfenden (1969). [d] Values are for complete conversion of reactant to product. [e] Computer calculated values with computed standard deviations.

Also listed in Table I for comparison is the K_i value of 4-hydroxypteridine, determined by double reciprocal plots of the rate of enzyme-catalyzed deamination of adenosine as a function of adenosine concentration in the presence and absence of 4-hydroxypteridine.

The dehydration of pteridine hydrate was also followed for a protracted period in the presence of enzyme. Following an initial burst (corresponding to approximately 50% conversion), disappearance of hydrate proceeded at a markedly reduced rate (Figure 7). The reverse reaction, pteridine hydration, showed similar behavior, proceeding with an initial burst followed by a slower phase. In both the hydration and the dehydration reactions, the rate of the post-burst phase, although slower than that of the initial burst, significantly exceeded (*ca.* 7-fold under the conditions of Figure 7) that of the nonenzymatic reaction, with a rate dependent on enzyme concentration. The ratio of *initial* rates of the fast to the slow reaction was ~7 for both hydration and dehydration under these conditions.

Compound **1**, which reversibly inhibits the deamination of adenosine with $K_i = 7.6 \times 10^{-7}$ M (Evans and Wolfenden, 1970), was also found to inhibit the rapid (*i.e.*, initial) phases of hydration and dehydration of pteridine–pteridine hydrate with $K_i = 7.7 \pm 1.9 \times 10^{-7}$ and $9.2 \pm 2.2 \times 10^{-7}$ M, respectively. This suggests that the pteridine reactions occur at the active site.

When introduced into the reaction mixture after completion of the burst, **1** (1.6×10^{-4} M) was found to inhibit the slow phases of the hydration and dehydration as well. These results confirm that the post-burst phase in each case is also subject to enzymic catalysis. From these results, it appeared

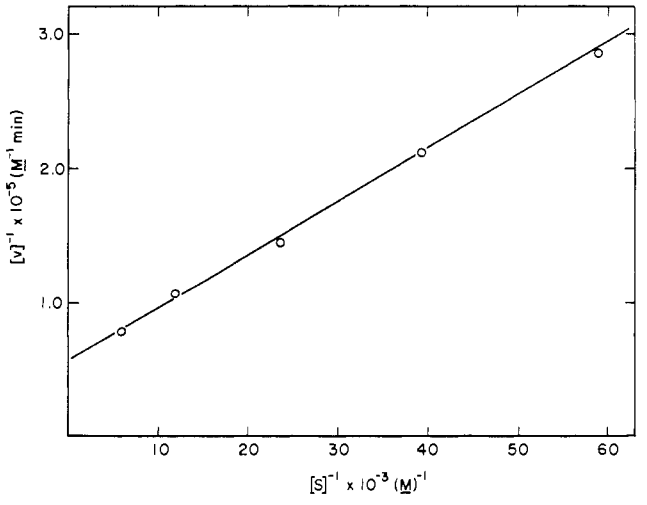


FIGURE 3: Double-reciprocal plot of the initial rate of adenosine deaminase catalyzed dehydration of pteridine hydrate in potassium phosphate buffer (0.1 M, pH 7.5), at 25° as a function of total pteridine hydrate concentration. Enzyme concentration is 3.5 μg/ml.

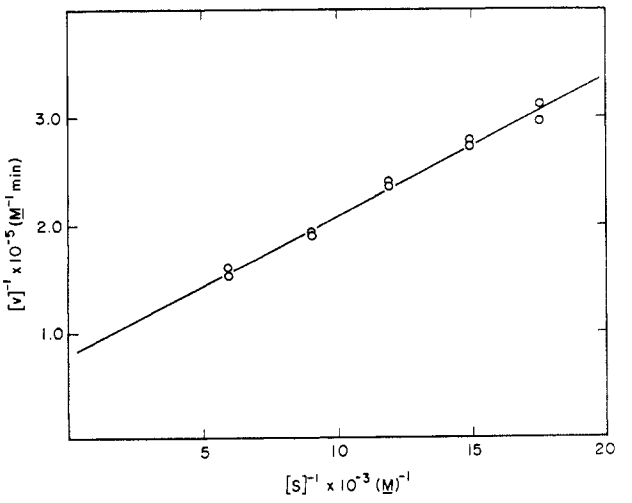


FIGURE 4: Double-reciprocal plot of the initial rate of hydration of pteridine catalyzed by adenosine deaminase (3.5 μg/ml) in 0.1 M potassium phosphate buffer (pH 7.5) at 25° as a function of pteridine concentration.

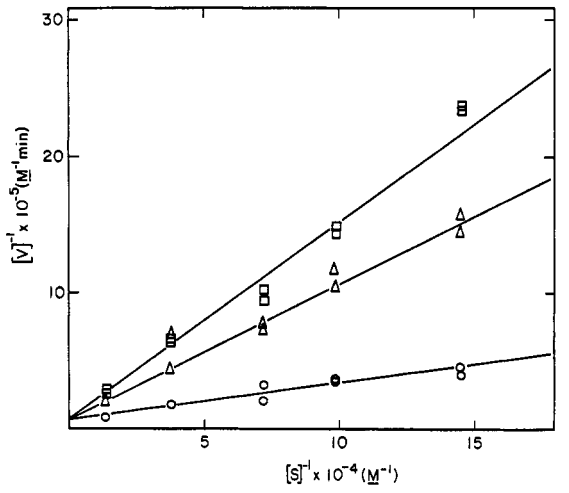


FIGURE 5: Double-reciprocal plot of the initial rate of enzyme-(0.065 μg/ml) catalyzed deamination of adenosine in 0.1 M potassium phosphate buffer (pH 7.5) at 25° as a function of substrate concentration. Individual plots are in the absence (○) and in the presence of pteridine hydrate (△, 0.0955 mM; □, 0.159 mM).

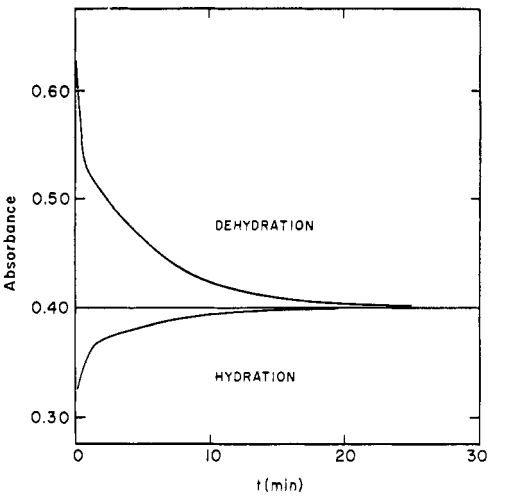


FIGURE 7: Spectrophotometric time-course curves (318 nm) for hydration of pteridine (lower curve) and dehydration of pteridine hydrate (upper curve) by calf duodenal adenosine deaminase in 0.1 M potassium phosphate buffer (pH 7.5) at 25°. In each case, total pteridine concentration is 5.8×10^{-5} M and enzyme concentration is 16.7 μg/ml. Ordinate is *total* solution absorbance.

likely that both enantiomers of pteridine hydrate were subject to enzymatic dehydration, the rate of one exceeding that of the other by a large factor.

This was supported by a series of polarimetric observations in which the optical rotation (365 nm) of a mixture of enzyme (100–700 μg/ml) and pteridine hydrate ($8–10 \times 10^{-3}$ M) was observed as a function of time. Under these conditions, pteridine hydrate produced a rapidly rising (positive rotation) deflection which decayed more slowly back to the zero rotation base line. From the reverse direction, anhydrous pteridine was enzymatically hydrated in the sample compartment of the spectropolarimeter (other conditions identical with those described above) with similar results. The deflection in this case, however, was toward *negative* rotation (Evans and Wolfenden, 1972).

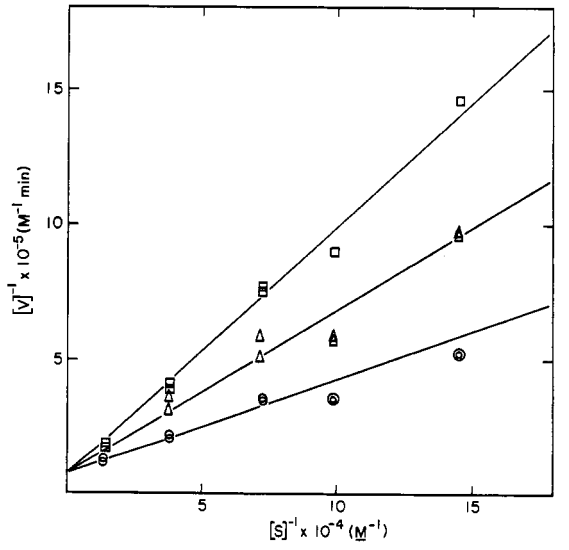


FIGURE 6: Double-reciprocal plot of the rate of adenosine deaminase (0.065 μg/ml) catalyzed deamination of adenosine in 0.1 M potassium phosphate buffer (pH 7.5) at 25° as a function of substrate concentration in the absence (○) and in the presence of anhydrous pteridine (△, 0.0805 mM; □, 0.134 mM).

Substitution of D_2O for water as solvent resulted in very minor changes in the rate of enzymatic hydration and dehydration of pteridine–pteridine hydrate, whereas effects on the nonenzymatic reaction were somewhat more pronounced (Table II). It has previously been noted that deuterium isotope effects on the hydrolytic activity of this enzyme are slight (Wolfenden, 1969; Orsi *et al.*, 1972).

The effect of high concentrations of enzyme on the apparent *equilibrium* between pteridine and its hydrate was examined by difference spectroscopy. The spectrum (400–200 nm) of equilibrated aqueous pteridine (35 μl of 1.36×10^{-3} M in 1.0 ml of distilled deionized water) was obtained with reference to water (a) and compared with a base-line spectrum of water *vs.* water (b). The difference spectrum a − b is shown in Figure 8 (curve 1). Calf duodenal adenosine deaminase was dialyzed against water (final resultant concentration 4.5 mg/ml, *ca.* 1.4×10^{-4} M) and its base-line spectrum (enzyme *vs.* enzyme) was determined (c). The pteridine solution (35 μl) described above was then added to 1.0 ml of this enzyme solution with mixing and the spectrum again determined (d). The difference spectrum d − c was computed and is shown in Figure 8 (curve 2). Each of the spectra described above was determined in duplicate and found to be reproducible.

TABLE II: Ratios of Enzymatic and Nonenzymatic Initial Reaction Velocities for Pteridine Hydration–Dehydration in H_2O and D_2O (0.1 M Potassium Phosphate Buffer, pH or pD 7.5 ± 0.5, 25°).[a]

Reaction	Nonenzymatic V_{iH_2O}/V_{iD_2O}	Enzymatic V_{iH_2O}/V_{iD_2O}
Pteridine hydration	1.53	0.89
Pteridine hydrate–dehydration	1.51	1.15

[a] Pteridine concentration is 1.0×10^{-4} M and enzyme concentration 6.7 μg/ml.

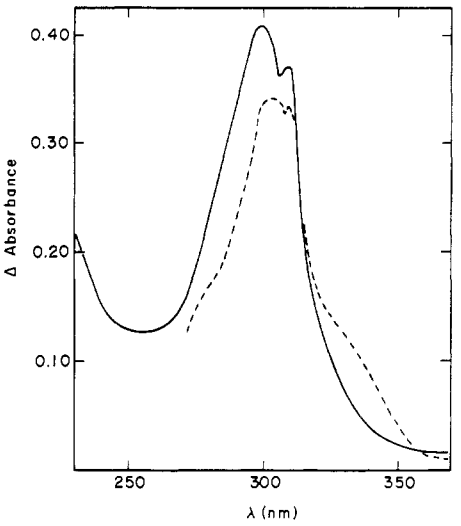
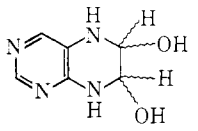


FIGURE 8: Uv difference spectra of equilibrated pteridine–pteridine hydrate (0.0476 mM) in the absence (curve 1, —) and presence (curve 2, ---) of calf duodenal adenosine deaminase (*ca.* 0.136 mM). All spectra are in distilled deionized water.

Pteridine 6,7-dihydrate (**7**), initially incorrectly identified



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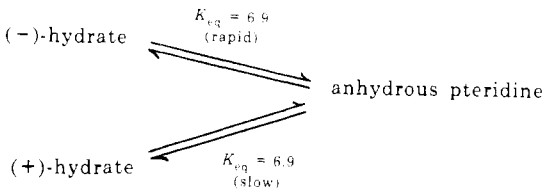
(Perrin, 1962; Inoue and Perrin, 1963) as a pyrazine derivative, is known to form in acidified aqueous solutions of pteridine (Albert *et al.*, 1966; Perrin, 1962; Inoue and Perrin, 1963). Its reported rate of formation ($t_{1/2}$ = 35 min at pH 2; Perrin, 1962) is several orders of magnitude below that of the 3,4-monohydrate (**6**) ($t_{1/2}$ = *ca.* 1 sec at pH 2; Perrin, 1962), and under the brief acid exposure conditions employed in the present work (3 sec), formation of **7** would not appear to be significant. To rule out the involvement of **7** in the spectral changes discussed above, the effect of enzyme on this compound was examined. When allowed to reach overall equilibrium, an acidified aqueous solution of pteridine contains both monohydrate **6** (21%) and dihydrate **7** (79%) (Albert *et al.*, 1966). For examination of the possible effect of enzyme on the dihydrate, an analytical wavelength (247 nm) was chosen which is isosbestic for the interconversion of monohydrate **6** and anhydrous pteridine **5** (Perrin, 1962; Figure 1, this work). At this wavelength and in pH 7.5 buffer (potassium phosphate, 0.1 M), the observed initial rate of reequilibration of pteridine dihydrate **7** at 25° (reported half-time at pH 7.58, *ca.* 100 min; Inoue and Perrin, 1963) was identical in the presence and absence of 17 μg/ml of calf duodenal adenosine deaminase.

Discussion

There is little doubt that the reactions described here are due to the activity of adenosine deaminase rather than a contaminant. Compound I is an extraordinarily effective inhibitor of all the observed reactions, and, as noted above, shows virtually identical K_i values as a competitive inhibitor of adenosine deamination and pteridine hydration by the calf duodenal enzyme. Table I shows that K_m values for

SCHEME II



$$K_{eq}\ (\text{app}) = \frac{(\text{anhydrous pteridine})}{(\text{total hydrate})} = 3.47$$

pteridine and pteridine hydrate are similar to their K_i values as inhibitors of the deamination of adenosine by the calf duodenal enzyme, and it has been previously found that this enzyme, obtained commercially, appears to be pure by physical criteria (Wolfenden *et al.*, 1968).

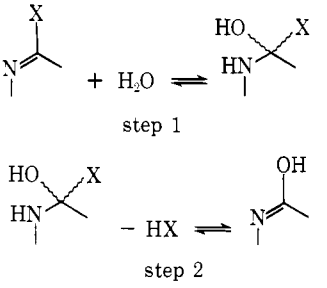
The present observations indicate that adenosine deaminase catalyzes attainment of equilibrium between pteridine and the levorotatory enantiomer of pteridine 3,4-monohydrate. During the dehydration of racemic pteridine hydrate, this enantiomer is consumed rapidly, leaving by default a net positive optical rotation due to the temporary excess of dextrorotatory (+)-enantiomer. In the reverse reaction, the levorotatory (−)-hydrate is synthesized rapidly and its negative rotation persists until final equilibrium is attained (Scheme II). In each case the rapid reaction of one enantiomer provides the spectrophotometric burst of *ca.* 50% conversion. Equilibration between pteridine and the dextrorotatory hydrate occurs more slowly, but is also subject to enzyme catalysis. This "slow" reaction occurs more rapidly in the presence than in the absence of enzyme, and is blocked by competitive inhibitors.

This is the first time that optical activity has been observed in pteridine monohydrate, chirality of which is required by the original structure assignment (Perrin, 1962). Both mammalian and fungal enzymes, which differ considerably in molecular weight and composition (Wolfenden *et al.*, 1968), catalyze stereospecific hydration and dehydration of pteridine–pteridine hydrate. This suggests that these enzymes have common mechanistic features, in accord with our previous observation that both enzymes are strongly inhibited by one diastereomer of **1** (Evans and Wolfenden, 1970).

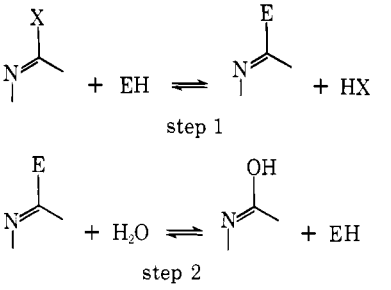
Enzymatic synthesis and dehydration of the levorotatory enantiomer of pteridine hydrate were rapid enough to permit an estimate of the kinetic constants (K_m and V_{max}) from initial reaction rates (Table I). Application of the Haldane relationship to the resulting values (Table I; mammalian enzyme) for K_m and V_{max} yields an apparent equilibrium constant of 6.45 for dehydration of the reactive (−)-enantiomer of pteridine hydrate, in reasonable agreement with a reported equilibrium constant (Inoue and Perrin, 1963) of 3.5 for nonenzymatic dehydration of *racemic* pteridine hydrate (see Scheme II). Less precise data for the enzyme from Takadiastase (Table I) yield an apparent equilibrium constant of 9.3 for the reactive hydrate enantiomer, still in fair agreement with the reported value.

Pteridine exhibits a higher K_m (and K_i) value than its (−)-hydrate (Table I). Figure 8 shows that the presence of enzyme induces in the pteridine spectrum a decided decrease in absorbance in the 300–310-nm region (region of intense absorption by anhydrous pteridine) and a corresponding increase in absorbance in the 320–350-nm range, the region

SCHEME III



SCHEME IV

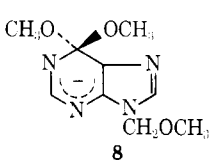


of intense absorption of the hydrate (Figure 1). The overall spectral changes, and the actual magnitude of the shift, appear to be consistent with the higher affinity of the enzyme for the hydrate implied by the K_i (and K_m) values.

The kinetic data summarized in Table I indicate that hydration of pteridine is carried on by the enzyme with an efficiency *equal to or exceeding* that exhibited in the deamination of 4-aminopteridine. This efficient and stereospecific hydration of pteridine by a "deaminase" is understandable if both hydration and deamination present a similar problem in catalysis, addition of water across the 3,4 double bond (Scheme III). When 4-aminopteridine is the substrate, ammonia is eliminated from the adduct (step 2). When pteridine is the substrate, elimination cannot occur, since it would involve cleavage of a stable C–H bond, and pteridine hydrate is the product.

An alternative double displacement mechanism (Scheme IV), previously considered for hydrolysis, would require displacement of the leaving group by enzyme, followed by displacement of the enzyme by water. This mechanism cannot easily explain the efficiency of the enzyme in catalyzing dehydration of pteridine hydrate.

Recent work by Liotta and Abidaud (1972) demonstrates that the proposed generation of a tetrahedral intermediate by nucleophilic attack at the 6 position of the purine ring is not only theoretically plausible, but experimentally observable under certain conditions. These workers found that methoxide ion, in strictly anhydrous media, adds to the 5,6 bond in the purine ring of 6-methoxy-9-methoxymethylpurine to give the short-lived (*ca.* 90 min) tetrahedral anionic σ complex **8** in



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what appears to be an explicit example of the process shown in step 1 of Scheme III.

Assuming that Scheme III is correct, one may question whether the transition state is reached during step 1 or step 2. Previous evidence suggests that, in hydrolytic reactions catalyzed by deaminases, breakdown of the tetrahedral intermediate is probably more rapid than its formation (Wolfenden, 1969, and references cited therein). In addition, more recent stopped-flow experiments in this laboratory have failed to provide any evidence for the accumulation of an enzyme-bound intermediate or product. Consistent with the possibility that the rate-determining step occurs before or during water attack (step 1), the overall limiting rates of hydration and deamination in the pteridine ring are of comparable magnitude, hydration being slightly the more rapid (Table I).

As in deamination reactions (Wolfenden, 1969; Orsi *et al.*, 1972), hydration of pteridine by adenosine deaminase shows a very slight solvent deuterium isotope effect, although water is a substrate. The near absence of an isotope effect suggests that in both cases the transition state may be reached before the scissile O–H bond in substrate water is appreciably stretched. There are of course many possible ambiguities in the interpretation of deuterium isotope effects, however the absence of an isotope effect in both reactions accords with the conclusion that the catalytic properties, and presumably the mechanisms, of deamination and hydration are similar.

The present results lead us to propose that covalent hydration occurs as the first step in enzymatic deamination of adenosine and 4-aminopteridine. The hydrates so formed are no doubt intrinsically unstable, since covalent hydration has never been observed in 4-aminopteridine or adenosine (in contrast with pteridine). Selective stabilization of such hydrates, implied by preferential binding of pteridine hydrate as compared with pteridine (Table I and Figure 8), may be one of the enzyme's principle functions in catalysis.

In summary, the present results support our earlier conclusion (Evans and Wolfenden, 1970), that adenosine deaminase catalyzes direct water attack on the substrate. Catalysis appears to be due, at least in part, to stabilization of a tetrahedral intermediate (*e.g.*, **2** and **4**) formed by water addition to the substrate. To our knowledge, this is the first-reported example of a hydrolytic enzyme which acts through a direct addition–elimination mechanism.

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Purification of the Polyenzymes Responsible for Tyrocidine Synthesis and Their Dissociation into Subunits†

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ABSTRACT: The biosynthesis of the antibiotic tyrocidine, a cyclic decapeptide →(NH_2)DPhe-Pro-Phe-DPhe(Trp,Tyr)Asn-Gln-Phe(Trp,Tyr)Val-Orn-Leu→ in extracts of *Bacillus brevis* (ATCC 8185), was shown to require three complementary enzyme fractions of mol wt 100,000, 230,000, and 440,000. As described (Roskoski *et al.*, *Biochemistry 9*, 4839, 4846 (1970)), these fractions activate the component amino acids with ATP to become thio ester linked to the enzymes. On combination, they polymerize sequentially, beginning from the N-terminal phenylalanine, in the direction indicated by the arrows to enzyme-bound peptidyl thio esters; addition of the last amino acid, leucine, causes release of cyclic decapeptide. The two larger enzyme fractions have now been purified to near homogeneity, and their enzymatic functions have been reassessed. The polyenzyme of mol wt 230,000 activates and binds, in addition to proline (*cf.* Roskoski *et al.*, *Biochemistry 9*, 4839, 4846 (1970)), L- and D-phenylalanine (*cf.* Kambe *et al.*, *J. Biochem.* (*Tokyo*) *69*, 1131 (1971)); the 440,000 molecular weight enzyme binds the last six amino acids in the sequence. Both polyenzymes contain 1 mol of pantetheine each; division of their molecular weights by the number of amino acids activated, *i.e.*, 230,000/3 and 440,000/6, yields uniform figures of 70,000–75,000. This seemed to indicate that the polyenzymes may be composed of amino acid activating subunits of such size, which is now confirmed by dissociation of the 230,000 and 440,000 molecular weight enzymes into subunits using two independent procedures. (1) Incubation of cell lysates with DNase for 40 min at 37° yields, on Sephadex G-200 chromatography or sucrose gradient centrifugation, a mixture of *ca.* 70,000 molecular weight fragment enzymes that respond to all amino acids present in the tyrocidine with ATP–PP_i exchange. (2) Sodium dodecyl sulfate gel electrophoresis causes partial dissociation of the 230,000 and 440,000 molecular weight enzymes to subunits of a similar size.

Our interest in the mechanism of biosynthesis of a group of bacterial antibiotic polypeptides was aroused by reports that such could take place in cytosol fractions that were exhaustively treated with RNase. We could confirm (Roskoski *et al.*, 1970a) that a ribosomal type of synthesis could be excluded, and that this synthesis involved polyenzymes which activated and bound the amino acids to be incorporated. The initial activation by ATP followed the scheme

$$\text{amino acid} + \text{ATP} \rightleftharpoons \text{AMP}\sim\text{amino acid} + PP_i$$

which is analogous to that in amino acid ligase activity. However, in contrast to the ligase reaction, the amino acid here is transferred to an enzyme-bound thiol to form an amino acid thio ester.

In the synthesis of gramicidin S, the first to be analyzed in detail, two complementary fractions are involved. Gramicidin S is a cyclic decapeptide composed of two identical pentapeptides, DPhe-Pro-Val-Orn-Leu, which cyclize head to tail. One enzyme fraction of mol wt 100,000 thioesterifies and racemizes phenylalanine, the second fraction of mol wt 280,000 thioesterifies the four amino acids following D-phenylalanine; these four can be bound without polymerization, and are initiated by reaction with the small enzyme that carries D-phenylalanine. Polymerization may be followed by the single addition of amino acids in sequence, which leads to the formation of enzyme-bound peptidyl thio esters of increasing chain length up to the pentapeptide. Addition of the fifth, the leucine, is rapidly followed by release of gramicidin S through cyclization; omission of an amino acid in the sequence stops polymerization. The size of the enzymes activating the amino acids appears to be approximately proportional to the number of amino acids activated. This was a first indication that we might be dealing here with polyenzymes composed of vectorially arranged amino acid specific binding subunits of similar molecular size.

Progressing to the study of tyrocidine biosynthesis, we hoped to find there an analogous mechanism on a larger scale. Tyrocidine, although a cyclic decapeptide like gramicidin S, is composed of a specific sequence of ten amino acids (Figure 1). Fujikawa *et al.* (1968) were the first to succeed in preparing from a tyrocidine-producing *Bacillus brevis* strain by DEAE-cellulose chromatography, two complementary cytosol fractions that synthesized the antibiotic; they isolated a 100,000 molecular weight enzyme analogous to the small one in gramicidin S that racemized and bound D-phenylalanine, and a larger fraction.

As reported (Roskoski *et al.*, 1970a), using ammonium sulfate fractionation and chromatography on Sephadex G-200, we obtained from a similar extract of *B. brevis* (ATCC 8185) three fractions of molecular weight 100,000, 230,000, and 460,000, and assumed that Kurahashi's heavier fraction

† From The Rockefeller University, New York, New York 10021. *Received August 18, 1972.* This study was supported by Grant GM-13972 from the U. S. Public Health Service, and by a fellowship (K. B.) from Deutsche Forschungsgemeinschaft.

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