Covalent Addition of Ammonia to Pteridine: the 3,4-Monoadduct

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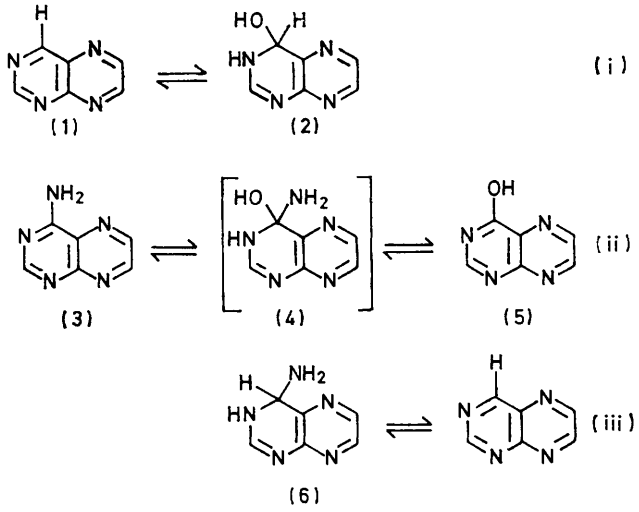
Covalent addition of ammonia to pteridine gives 4-amino-3.4-dihydropteridine, characterized by the stoichiometry of its formation and by direct n.m.r. observation. The susceptibility of the molecule to adenosine aminohydrolase is considered.

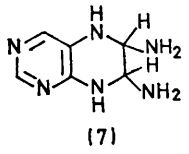
CATALYSIS of covalent hydration of pteridine (1) [equation (i)] by adenosine aminohydrolase has been demonstrated recently.[1,2] Close kinetic similarities between this process and enzyme-catalysed deamination



SCHEME 1

of 4-aminopteridine (3) led us to suggest that the two reactions are mechanistically similar. On this basis the enzymic deamination should proceed through an initial covalent water addition [equation (ii)], and the hydration (i) serves as a mechanistic analogue of this process. Continuing investigation of this phenomenon led to our interest in elimination of ammonia from 4-amino-3,4-dihydropteridine (6) [equation (iii)], which is similarly analogous to the second stage in deamination, loss of ammonia from the hypothetical tetrahedral intermediate (4).

4-Amino-3,4-dihydropteridine (6), the covalent 3,4-monoadduct of ammonia to pteridine, has not been reported previously. On the contrary, studies by Albert and Ohta[3] indicated that pteridine in aqueous



(7)

ammonia formed the diadduct (7) exclusively, as determined by both u.v. and n.m.r. spectroscopy. This paper describes the first observation of the adduct (6). It also makes available a reaction [equation (iii)] of mechanistic consequence in our study of adenosine aminohydrolase and provides a novel illustration of an equilibrium mixture of five separate components, all visible simultaneously under readily accessible conditions.

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‡ Failure of this criterion in the case of addition of methylamine to pteridine[3] indicates its less than conclusive nature.

RESULTS

Spectra.—An ammonia buffer was prepared by (careful) addition of concentrated hydrochloric acid to concentrated ammonia solution (to pH 10·0). This buffer was 4·7M in free ammonia (by titration).

When pteridine in aqueous sodium hydrogen carbonate buffer (0·1M; pH 10·0) was treated with successive portions of the ammonia buffer, rapid u.v. spectral changes occurred, with the spectrum assuming an increasing resemblance to that of the 3,4-monohydrate (2).[4] The absorbance increased in the 320 and 265 nm regions, and decreased simultaneously near 300 nm; isosbestic points occurred at 313, 278, and 243 nm. Such spectral similarity with the 3,4-monohydrate (2) is one potential criterion ‡ for identification of the 3,4-monoadduct (6). The initially observed isosbestic points fail at higher ammonia concentrations ($\geqslant$*ca.* 1M).

Stoicheiometry.—The foregoing experiment was repeated and the absorbance change evaluated quantitatively as a function of added ammonia. Thus, to equilibrated pteridine–pteridine hydrate (2·5 ml; $9{\cdot}3 \times 10^{-5}$M total pteridine) in 0·1M-sodium hydrogen carbonate buffer (pH 10·0), measured samples of the ammonia buffer were added, and the resulting absorbance decrease at 300 nm was determined (1 cm cuvette; 22 °C). For a 1 : 1 addition, the relationship between absorbance change (ΔA) and ammonia concentration is given by equation (iv), whereas for a 2 : 1 stoicheiometry the appropriate expression is equation (v). In each equation, C_0 is the initial total

$$1/[NH_3] = K_1C_0\Delta\varepsilon/\Delta A - K_1 \qquad \text{(iv)}$$

$$1/[NH_3]^2 = K_2C_0\Delta\varepsilon/\Delta A - K_2 \qquad \text{(v)}$$

pteridine concentration, $\Delta\varepsilon$ the difference in molar extinction coefficient between reactant and product (at 300 nm), and K_n the equilibrium constant for formation of an n : 1 adduct. Based on these equations, two double reciprocal plots of the absorbance/concentration data were constructed, one (Figure 1A) of $[NH_3]$ and one (Figure 1B) of $[NH_3]^2$ against observed u.v. absorbance change (corrected for dilution). Only the plot in Figure 1A provided the predicted straight line and a 1 : 1 stoicheiometry is thus indicated, with K_1 1·0 l mol^{-1}.

At the highest ammonia concentrations, there is a suggestion of deviation from linearity in Figure 1A.

[1] B. Evans and R. Wolfenden, *J. Amer. Chem. Soc.*, 1972, **94**, 5902.
[2] B. E. Evans and R. V. Wolfenden, *Biochemistry*, 1973, **12**, 392.
[3] A. Albert and K. Ohta, *J. Chem. Soc.* (*C*), 1971, 2357.
[4] D. D. Perrin, *J. Chem. Soc.*, 1962, 645.

Failure of the isosbestic points in approximately the same region suggests this deviation may be a result of diadduct formation. N.m.r. observations confirm this supposition.

N.m.r. Spectra.—N.m.r. spectra of pteridine were obtained in the solvents listed in Figure 2. All spectra were re-scanned to assure attainment of at least a temporarily stable state in each solution. Pteridine is degraded by alkali (0·1N-KOH), although at a relatively low rate.[5] The spectrum of pteridine in N-NaOH was therefore determined within 3 min after dissolution. The resonances shown [Figure 2, (I)] are most likely due to the anion of hydrate (2), as they revert virtually completely to the

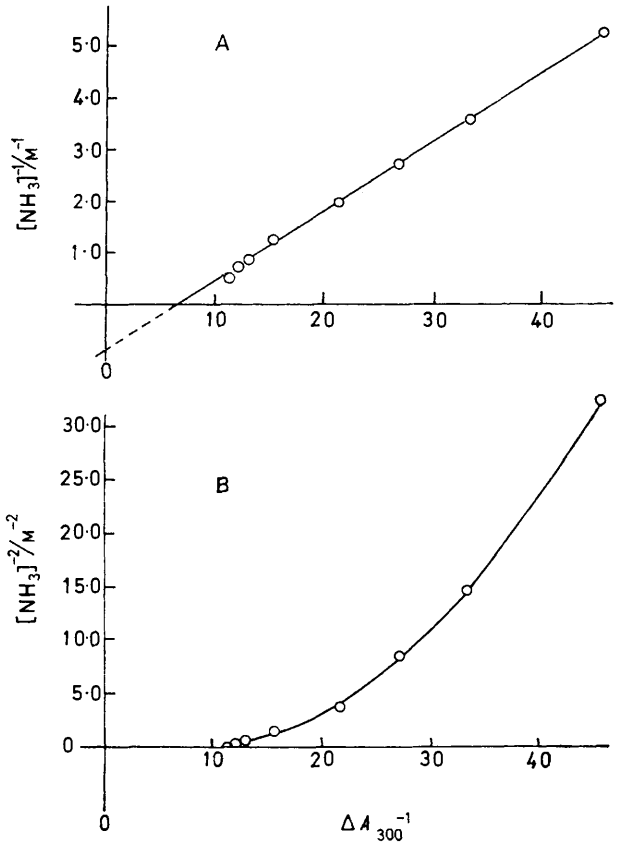


FIGURE 1 Double reciprocal plots of ammonia concentration {A, $[NH_3]$; B, $[NH_3]^2$} *vs.* resulting absorbance change (300 nm, corrected for dilution) in a solution of equilibrated pteridine–pteridine hydrate (pH 10·0)

' normal ' spectrum of aqueous pteridine upon neutralization (HCl) to pH *ca.* 7. The results of all the spectral scans (artist's conceptions) are shown in Figure 2.

The spectra in pH 10·0 sodium hydrogen carbonate buffer and concentrated (unbuffered) ammonia [(II) and (III)] agree with earlier results. Spectrum (II) shows the resonances due to both anhydrous pteridine (1) and pteridine 3,4-monohydrate (2). These absorbances agree with those previously reported,[6] and confirm the 3·5 : 1 equilibrium distribution between the anhydrous form and the 3,4-monohydrate in aqueous medium at this pH.[4,7]

In concentrated (unbuffered) ammonia [spectrum (III)] the low-field spectrum of pteridine shows only the two singlets (τ 2·0 and 2·3, each 1H) characteristic of the ammonia diadduct, in agreement with the observation of Albert and Ohta.[3] Diluted to 1M-ammonia [spectrum (IV)], this unbuffered sample shows, in addition to the ammonia diadduct, absorbances due to anhydrous pteridine, the 3,4-monohydrate, and the anion of the monohydrate [*cf.* spectra (I) and (II)]. The spectrum appears to be a superposition of spectra (I)—(III) with virtually no extraneous peaks. The ammonia monoadduct (6) is not evident.

In the concentrated buffered ammonia, such extraneous peaks do appear [spectrum (V) was determined in buffered deuterioammonia, pD 10·0; it is identical with that observed in buffered ammonia, but allows access to the resonances near the water absorbance]. In this solvent, the low-field region is complex, showing at least eight peaks between τ 1·5 and 2·5. The two singlets of approximately equal intensities at τ 1·95 and 2·3 indicate a significant level of the ammonia diadduct—the integrated intensities suggest *ca.* 3·3 times the monoadduct level.

The remaining peaks in this region are a singlet at τ 1·5, a multiplet at 2·05, and another multiplet at 2·35 (intensities *ca.* 1 : 1 : 1). These peaks resemble superficially those of the 3,4-monohydrate. That they are not in fact due to the hydrate is shown both by the very low level of the anhydrous pteridine absorbances (τ 0—1), which must accompany those of the hydrate in a 3·5 : 1 ratio in this medium (see before), and by the effect of six-fold dilution with water (with D_2O in the case of ND_3 buffer). The latter treatment partially reverses the ammonia addition and brings out the resonances of anhydrous pteridine and its 3,4-monohydrate for specific comparison. The τ 1—3 resonances in spectrum (V) are assigned to H-2, H-6, and H-7 of the 3,4-ammonia monoadduct (6), by analogy with the corresponding similar assignments to the 3,4-monohydrate (2) [6] and the 3,4-methanol adduct.[8]

In concentrated ND_3 buffer, the spectrum is identical with that obtained in NH_3 buffer (see before), but the high-field region is also accessible, showing doublets due to H-6 and H-7 of the diadduct (τ 5·25 and 5·65; *cf.* the assignments to the methylamine diadduct,[3] τ 6·05 and 6·17, and the methanol diadduct,[8] 5·21 and 5·38) and to the monoadduct H-4 (τ 4·3; *cf.* methanol monoadduct,[8] 4·01, and monohydrate, 3·81).

In the diluted ammonia buffer, 0·8M in free ammonia, there appear to be five separate species observable; anhydrous pteridine (1), the 3,4-monohydrate (2), its anion, the 3,4-ammonia monoadduct (6), and the 5,6,7,8-ammonia diadduct (7), all in significant concentrations.

DISCUSSION

The u.v. spectral changes and n.m.r. absorptions indicate that pteridine and dilute (<1M) ammonia react at pH 10 to give primarily the 3,4-ammonia monoadduct (6), with K_1 1·0 l mol^{-1}. In more concentrated ammonia (>1M), at pH 10, the diadduct (7) is increasingly favoured, with K_2 *ca.* 0·7 l^2 mol^{-2} (from integrated n.m.r. intensities).

Earlier failure to observe the ammonia monoadduct (6) in unbuffered ammonia was most likely a result of the high basicity of this medium. At the intermediate ammonia concentrations at which monoadduct form-

[5] A. Albert and H. Yamamoto, *J. Chem. Soc.* (*C*), 1968, 2289.
[6] J. W. Bunting and D. D. Perrin, *J. Chem. Soc.* (*B*), 1966, 433; see also A. Albert, T. J. Batterham, and J. J. McCormack, *ibid.*, p. 1105.
[7] Y. Inoue and D. D. Perrin, *J. Chem. Soc.*, 1963, 2648.
[8] A. Albert and H. Mizuno, *J. Chem. Soc.* (*B*), 1971, 2423.

ation predominates, the pH of aqueous ammonia exceeds the pK_a (11·21)[4] of pteridine hydrate (2), resulting in formation of the hydrate anion as a shunt for the available pteridine. Buffering permits exposure of free pteridine to significantly higher concentrations of free ammonia without exceeding the pK of the hydrate.

The results described are depicted in Scheme 2. The cycle on the right-hand side of this Scheme permits evaluation of the equilibrium constant for hydroxide ion addition to pteridine (K_{OH^-} 180 l mol^{-1}) from the values of the other three constants. Together with the equilibrium constants for 3,4-addition of ammonia (1·0 l mol^{-1}; this work), water (0·0052 l mol^{-1}),[4,7]

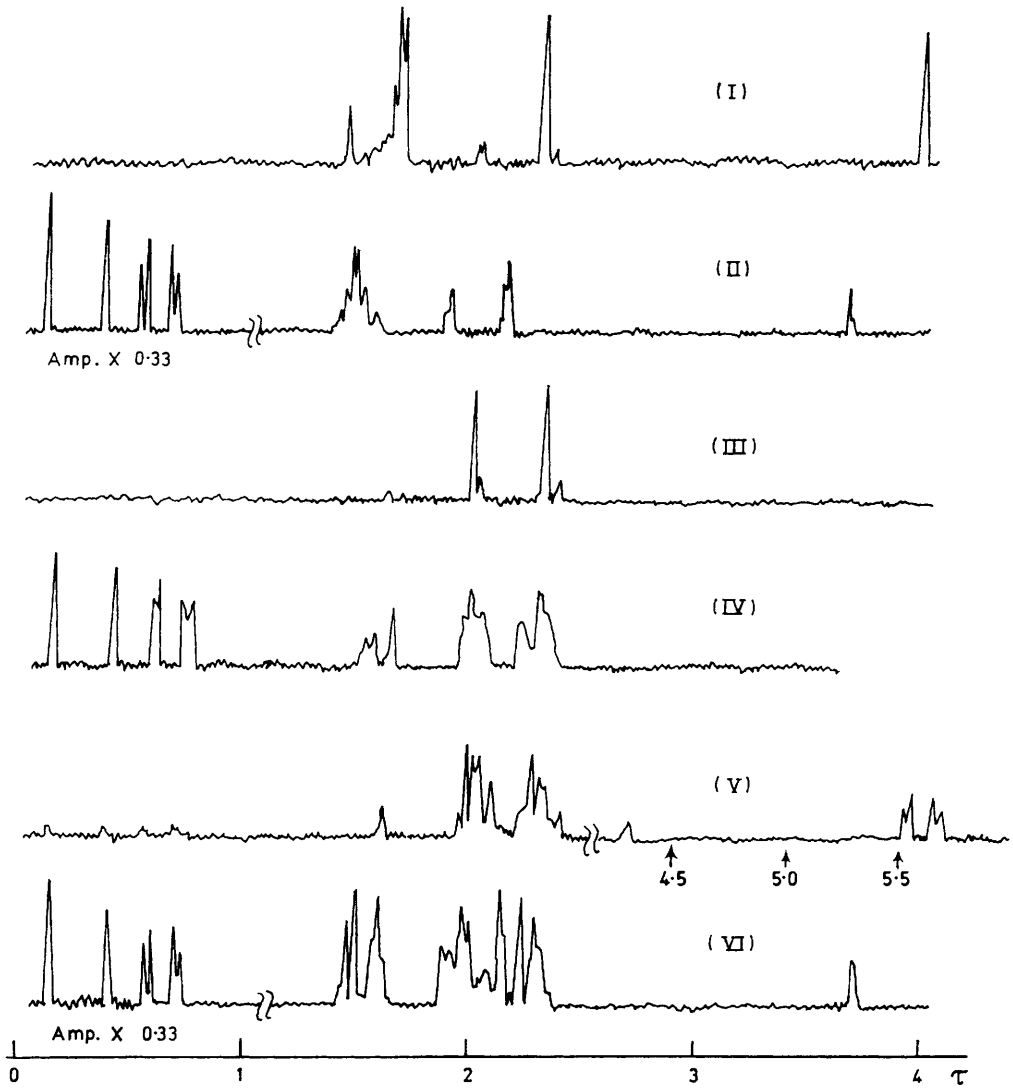


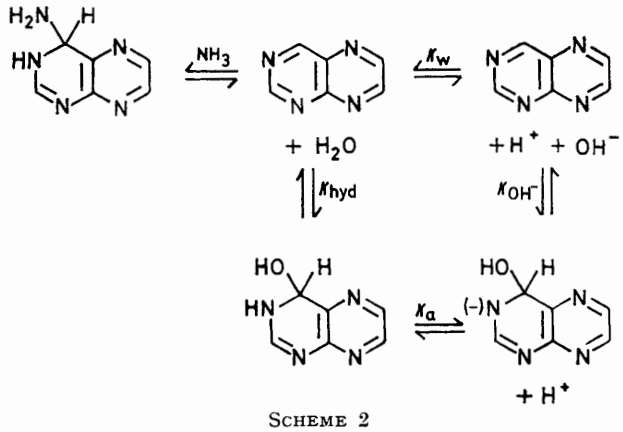
FIGURE 2 Artist's conceptions of n.m.r. spectra of pteridine obtained in (I) N-sodium hydroxide; (II) 0·1M-sodium hydrogen carbonate buffer, pH 10·0; (III) concentrated unbuffered aqueous ammonia; (IV) dilute (1M) unbuffered aqueous ammonia; (V) concentrated buffered deuterioammonia (pD 10·0; 4·7M in free ND_3); (VI) dilute buffered deuterioammonia (pD 10·0; 0·8M in free ND_3)

Structural similarity between the hydrate (2) and the ammonia monoadduct (6) suggests a similar pK for the latter, which might complicate such an explanation. However, a plot of the observed initial rate of deamination of adduct (6), in a series of buffers, *vs.* pH is essentially flat from pH 9 to 12. This suggests that the pK_a of the ammonia adduct (6) lies above 12, *i.e.* well above that of the hydrate. The anion of the ammonia adduct apparently does not contribute to the equilibria observed in this study.

and methanol (*ca.* 0·04 l mol^{-1}),[8] this value indicates an order of effectiveness as pteridine addends of 34,500 : 192 : 7·8 : 1 for OH^-, NH_3, MeOH, and H_2O, roughly in line with the pK_a values of the corresponding acids (H_2O, NH_4^+, $MeOH_2^+$, H_3O^+).

A search for adenosine aminohydrolase activity on adduct (6), proved negative, a result of mechanistic consequence. For catalysis of dehydration of pteridine hydrate (2) by adenosine aminohydrolase, $V_{max.}$ is 5 μmol min^{-1} (mg enzyme)$^{-1}$.[1,2] The observed rate of

non-enzymic deamination of the ammonia 3,4-adduct (6) in 1M-potassium phosphate buffer (pH 7·5) is described by the first-order rate constant k_{obs} 0·0173 s^{-1} (at 22 °C). Experimentally, no acceleration of this



SCHEME 2

$K_w = 1{\cdot}8 \times 10^{-16}$ l mol^{-1}; $K_{hyd} = 5{\cdot}2 \times 10^{-3}$ l mol^{-1}; $K_a = 6{\cdot}3 \times 10^{-12}$ l mol^{-1}

deamination by adenosine aminohydrolase is observed (3×10^{-4}M substrate, 0·5 mg ml^{-1} adenosine aminohydrolase).

These facts imply that the ammonia adduct (6) is at least ten-fold less effective a substrate for the enzyme. Catalysis appears substantially more efficient in the dehydration reaction. This result supports our earlier conclusion [1,2] that the principal catalytic influence of adenosine aminohydrolase is exerted early in the overall deamination, at least prior to formation of the tetrahedral intermediate (4) [equation (ii)], and probably early within the first stage shown in (ii) and mimicked by the hydration (i).

EXPERIMENTAL

Adenosine aminohydrolase (adenosine deaminase, E.C. 3.5.4.4) was obtained from Boehringer-Mannheim Corp. as a suspension (10 mg ml^{-1}) in 2·8M-ammonium sulphate which was used directly in enzyme assays.

Enzyme activities were determined spectrophotometrically.[1,2] These procedures, as well as determination of u.v. spectral information, were carried out on a Coleman/Perkin-Elmer 124 spectrophotometer. N.m.r. spectra were determined with a JEOL C60HL instrument (ambient temperature).

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