502. *Pteridine Studies. Part XX.*[1] *Reversible Water Addition to Hydroxypteridines.*

By Y. Inoue and D. D. Perrin.

Reversible covalent hydration of 2-hydroxypteridine across the 3,4-double bond to form 3,4-dihydro-2,4-dihydroxypteridine strongly favours the hydrated species: at 20° the equilibrium ratio is 320 : 1. Similarly, for the formation of 7,8-dihydro-6,7-dihydroxypteridine from 6-hydroxypteridine, the ratio is 125 : 1. Anion formation leads to loss of the bound water. Neither 4- nor 7-hydroxypteridine shows this type of reaction. Xanthopterin (2-amino-4,6-dihydroxypteridine) adds water across the 7,8-double bond.

Acid dissociation constants, equilibrium constants, and ultraviolet spectra of hydrated and anhydrous species are recorded for 2- and 6-hydroxypteridines and some derivatives, including 2,6- and 4,6-dihydroxypteridines and xanthopterin.

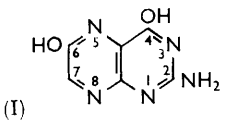
Large differences in the rates of oxidation of mono- and di-hydroxypteridines in the presence of xanthine oxidase can be explained if the hydrated species are resistant to enzymic oxidation.

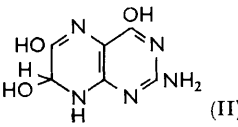
Xanthopterin (2-amino-4,6-dihydroxypteridine) has been shown (by the changes, with time and hydrogen-ion concentration, in its fluorescence and ultraviolet spectra) to exist

[1] Part XIX, Albert, Howell, and Spinner, preceding paper.

in aqueous solution in equilibrium between two forms, only one of which is oxidised to leucopterin (2-amino-4,6,7-trihydroxypteridine) in the presence of xanthine oxidase.[2] Potentiometric back-titration of alkaline xanthopterin solutions produces pH drifts similar to those observed for 6-hydroxypteridine,[3] where they are due to reversible water-addition across the 7,8-double bond to form 7,8-dihydro-6,7-dihydroxypteridine.[4–6] This suggests that the transformation of xanthopterin is between the species (I) and (II), and not, as originally postulated,[2] between an enol and a keto-form.

Recently, using rapid pH-titration and spectrophotometric methods, we have found reversible water-addition to occur to some substituted pteridines and triazanaphthalenes [7] and also across the 3,4-double bond of pteridine.[8] The study with pteridine included the


(I)


(II)

isolation of salts of the " hydrated " pteridine species, 3,4-dihydro-4-hydroxypteridine, and of the corresponding ring-opened formylpyrazine derivative. The hysteresis loop observed in the acid–base titration of 2-hydroxypteridine [7] confirmed earlier conclusions [5,9] that in neutral aqueous solution 2-hydroxypteridine is hydrated across the 3,4-double bond. A detailed study has now been made of the equilibria, the acid–base properties, and the ultraviolet spectra of a number of hydroxypteridines, including xanthopterin, which have been found to show this hysteresis effect. There seems no doubt that, in 2-hydroxypteridine and its derivatives, reversible water-addition occurs across the 3,4-double bond of the pteridine molecule, and, similarly, that in 6-hydroxypteridine and its derivatives water-addition occurs across the 7,8-double bond. As discussed below, this hydration equilibrium appears to explain much of the variation observed in the rates at which pteridine and some of its hydroxy-derivatives are oxidised in the presence of xanthine oxidase.[10]

Experimental

Materials.—We are indebted to Professor A. Albert for generously providing all the pteridine derivatives used in this study.

Methods.—Potentiometric titrations were carried out with magnetic stirring and a recording pH-meter assembly as described previously; [11] a micrometer syringe was used to add 0·100M-acid or -alkali in steps of one-tenth equivalent. Total titration times were around 3 minutes, which was much less than the time needed for appreciable interconversion of " anhydrous " and " hydrated " species such as 6-hydroxypteridine $\rightleftharpoons$ 7,8-dihydro-6,7-dihydroxypteridine. At equilibrium the " hydrated " species (HY), which is the weaker of the two acids, tends to predominate in neutral solution in those cases where hydration occurs, whereas the anion of the " anhydrous " species (HX) is the preferred form in alkaline solution. Hence, a hysteresis loop was observed when a neutral solution was titrated rapidly with alkali, allowed to stand, and then back-titrated with acid. From these curves, together with the curve obtained when the titration was carried through sufficiently slowly for equilibrium to be reached after each addition, the acid dissociation constants of the two species and their equilibrium ratios as anions and as neutral molecules were calculated as follows:

By means of the Henderson equation, the alkali-titration curve provided an initial estimate of pK_a^Y, and the acid titration curve gave an approximate value for pK_a^X. Similarly, pK_a^{eq}

[2] Schou, *Arch. Biochem.*, 1950, **28**, 10.
[3] Albert, Brown, and Cheeseman, *J.*, 1952, 1620.
[4] Albert, *J.*, 1955, 2690.
[5] Brown and Mason, *J.*, 1956, 3443.
[6] Albert and Reich, *J.*, 1961, 127.
[7] Perrin and Inoue, *Proc. Chem. Soc.*, 1960, 342.
[8] Perrin, *J.*, 1962, 645.
[9] Albert and Howell, *J.*, 1962, 1591.
[10] Bergmann and Kwietny, *Biochem. Biophys. Acta*, 1959, **33**, 29.
[11] Perrin, *J.*, 1960, 3189.

was obtained from the middle curve, where $K_a^{eq} = a_{H^+}([X^-] + [Y^-])/([HX] + [HY])$. If $K_1 = [Y^-]/[X^-]$ and $K_2 = [HY]/[HX]$, this expression can be transformed into:

$$pK_a^{eq} = pK_a^X + \log (1 + K_2)/(1 + K_1)$$
$$= pK_a^Y - \log K_2(1 + K_1)/K_1(1 + K_2).$$

The values of K_1 and K_2 obtained from these equations were used to correct the initial estimates of pK_a^X and pK_a^Y for the (usually low) concentrations of the other species also present. Repetition of this process led finally to the values of the constants listed in Table 1. In the particular case that $K_1 \ll 1$, and $K_2 \gg 1$, calculation was facilitated by using the approximations, $-\log K_1 \simeq pK_a^Y - pK_a^{eq}$, and $\log (1 + K_2) \simeq pK_a^{eq} - pK_a^X$. Equations similar to the above were derived for analysing the titration curves of the dihydroxypteridines.

TABLE 1.

Acid dissociation constants and equilibrium ratios of " hydrated " (Y) and " anhydrous " (X) hydroxypteridines, at 20°.

Pteridine derivative	pK_{a1}^X	pK_{a2}^X	pK_{a1}^{eq}	pK_{a2}^{eq}	pK_{a1}^Y	pK_{a2}^Y	[Y]/[X]	$[Y^-]/[X^-]$	$[Y^{--}]/[X^{--}]$
2-OH	7·7		10·15		11·05		320	0·14	
2-OH-4-Me	8·2		9·00		10·85		6	0·014	
2-OH-6-Me	7·95		10·05		11·00		110	0·10	
2-OH-7-Me	8·07		9·60		10·85		35	0·058	
2-OH-6,7-Me_2 ...	7·95		9·80		11·15		70	0·046	
2-OH-4,6,7-Me_3	8·5		8·65		11·5		0·42	0·0004	
6-OH	6·45		8·55		9·90		125	0·045	
6-OH-2-Me	6·53		8·50		10·05		80	0·028	
6-OH-4-Me	6·3		8·20		10·0		75	0·016	
6-OH-7-Me	7·09		7·46		10·0		0·87	0·001	
2-SH	6·52		9·00		9·72		380	0·24	
2,6-$(OH)_2$	5·99	9·66	8·40	9·80	8·84	10·29	400	0·57	0·14
4,6-$(OH)_2$	6·05	9·51	6·40	9·51	8·34	10·08	1·24	0·006	0·0017
4,6-$(OH)_2$-2-NH_2	6·59	9·31	6·88	9·32	8·65	9·99	1·01	0·009	0·0019

The following show no hysteresis effect:

Deriv.	4-OH	4-OH-2-NH_2	7-OH	2,4-$(OH)_2$	2,7-$(OH)_2$	4,7-$(OH)_2$	6,7-$(OH)_2$
pK_{a1}^X	7·89	7·92	6·41	7·91	5·85	6·08	6·87
pK_{a2}^X					9·94	9·62	10·0

Ultraviolet spectra were recorded on either a Perkin–Elmer " Spectracord " or a Shimadzu model RS 27 recording spectrophotometer, into the cell compartments of which was fitted a 1-cm. cell attached to a modified Chance [12] rapid-reaction apparatus. In this apparatus, which could be used both for stopped-flow and for continuous-flow studies, the time taken between mixing of the reactant solutions in the mixing chamber and placing them in the cell was close to 1 second. Where species were relatively stable, the extinction coefficients and maxima were checked on a Hilger " Uvispek " spectrophotometer.

RESULTS

Fig. 1 shows the hysteresis loop observed at 20° in the rapid acid–base titration of xanthopterin, and also the titration curve obtained under equilibrium conditions. Table 1 summarises the results for several hydroxy- and dihydroxy-pteridines. In Fig. 2, the ultraviolet spectra of the neutral molecules existing in xanthopterin solutions and of the anhydrous dianion are plotted: the spectra of the two monoanions were not obtained and the spectrum of the hydrated dianion resembles that of the neutral molecule (except that there is no inflection near 300 mμ). Table 2 records absorption maxima and extinction coefficients for " hydrated " and " anhydrous " forms of some of the substances listed in Table 1.

Where reversible hydration could be detected, kinetic studies (to be reported in detail elsewhere) have been made and the constants, k_h and k_d, have been obtained for the rate equation,

$$-d([X^-] + [HX])/dt = k_h([X^-] + [HX]) - k_d([Y^-] + [HY]).$$

[12] Chance, in " Rates and Mechanisms of Reactions," Technique of Organic Chemistry, Vol. VIII, ed. Friess and Weissberger, Interscience Publ., Inc., New York, 1953, p. 690.

In all cases the reaction was acid–base catalysed so that the rate constant, k_h, for the hydration was a composite one given by

$$k_h([X^-] + [HX]) = k_1[H^+][HX] + k_2[HX] + k_3[OH^-][HX] + k_4[H^+][X^-] + k_5[X^-] + k_6[OH^-][X^-],$$

in which the constants k_2, k_3, k_4, and k_5 could not be separately evaluated. Typical values of log k_1 at 20° and $I = 0{\cdot}1$, obtained from the pH-dependence of log k_h in the region, pH 4·2—5·2, are 4·76 for 2-hydroxy-, 4·71 for 2-mercapto-, and 3·20 for 6-hydroxy-pteridine. For 2,6- and 4,6-dihydroxypteridine, log k_1 is 4·25 and 2·23, respectively. These values are probably related

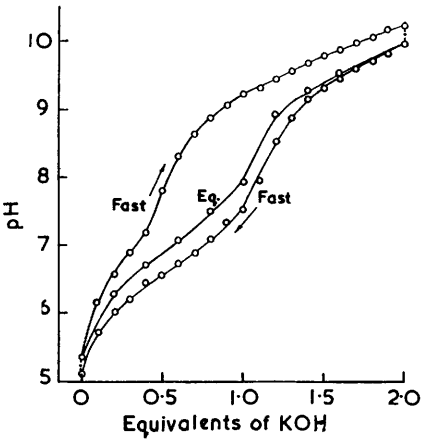


FIG. 1. Hysteresis loop in rapid titration of 0·001M-xanthopterin with 0·1M-potassium hydroxide and back-titration with hydrochloric acid, and the equilibrium titration curve.

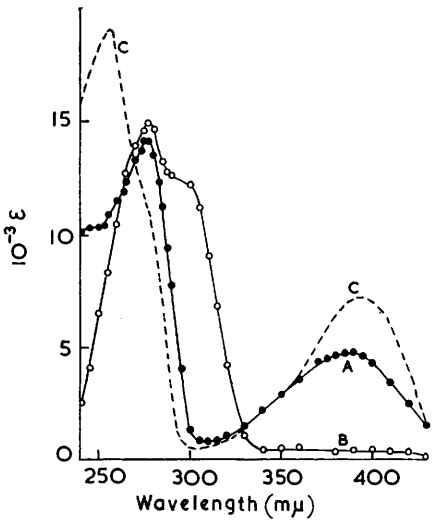


FIG. 2. Ultraviolet spectra of xanthopterin species in water. (A) "Anhydrous" neutral molecule; (B) "hydrated" neutral molecule (7,8-dihydro-7-hydroxyxanthopterin); (C) anhydrous dianion.

to the ease with which the activated complex is formed, and this, in turn, is believed to be the rate-determining step in the reaction.

DISCUSSION

The conclusion that covalent hydration of 6-hydroxypteridine and its derivatives occurs across the 7,8-double bond is supported by the hindering effect exerted by a 7-methyl group.[6] This is reflected in the equilibrium ratio of the neutral hydrated and anhydrous species in aqueous solution, which is very much smaller for 6-hydroxy-7-methylpteridine than for 6-hydroxypteridine and its 2- and 4-monomethyl derivatives. Similarly the present work confirms the view that in 2-hydroxypteridine hydration takes place across the 3,4-double bond, as originally postulated by Brown and Mason:[5] this is also supported by the hindering effect exerted by a 4-(but not a 6- or 7-)methyl group.[9] A factor which is believed to facilitate this type of reaction is that neutral monohydroxypteridines exist predominantly as the amide (lactam) tautomers such as (III), rather than the enols (lactims) such as (IV).[5] This tautomerism, involving as it does only a proton transfer between a nitrogen and an oxygen atom, should be much too fast to be detected by our experimental techniques.

Formation of the amide tautomer leads to loss of aromatic character (of the pyrimidine ring in 2- and of the pyrazine ring in 6-hydroxypteridine) and the emergence of an isolated

TABLE 2.

Light-absorption spectra of " hydrated " (Y) and " anhydrous " (X) hydroxypteridines in water.

Pteridine derivative	Form	pH	$\lambda_{max.}$ (mμ) with log ε in parentheses
2-OH	X	5·6	353 (3·84)
	X^-	11·8	224 (4·31); 265 (3·82); 375 (3·83)
	Y	7·3	232 (3·96); *281* (3·57); 308 (3·89)
	Y^-	(0·1M-NaOH)	230 (3·78); 267 (3·69); 312 (3·66); *340* (*3·38*)
2-OH-7-Me	X	5·6	352 (4·01)
	X^-	11·8	226 (4·33); 263 (3·81); 371 (3·92)
	Y	7·3	235 (3·92); *279* (*3·48*); 310 (3·98)
	Y^-	(0·1M-NaOH)	234 (3·92); 274 (3·65); 315 (3·89); *344* (3·53)
2-OH-6,7-Me_2	X	5·6	235 (3·64); 355 (4·01)
	X^-	11·8	228 (4·32); 263 (3·78); 374 (3·90)
	Y	7·3	238 (3·88); *281* (*3·32*); 315 (3·94)
	Y^-	(0·1M-NaOH)	234 (3·88); 268 (3·60); 317 (3·89)
6-OH	X	4·6	237 (3·75); 335 (3·73)
	X^-	12	222 (4·27); 256 (3·88); 358 (3·74)
	Y	6·0	288 (3·99)
	Y^-	11·3	236 (3·39); 294 (4·03)
6-OH-2-Me	X	4·6	235 (3·89); 345 (3·77)
	X^-	11	222 (4·31); 254 (3·97); 362 (3·76)
	Y	6·0	285 (4·02)
	Y^-	11·3	293 (4·07)
6-OH-4-Me	X	4·6	240 (3·88); 338 (3·79)
	X^-	11	223 (4·27); 259 (3·92); 357 (3·82)
	Y	6·0	287 (4·13)
	Y^-	11	235 (3·49); 289 (4·07)
6-OH-7-Me	X	4·6	237 (3·74); 330 (3·84)
	X^-	11·6	225 (4·36); 257 (3·89); 348 (3·95)
	Y	6·8	288 (4·09)
	Y^-	11·6	*263* (*3·79*); 288 (3·98)
4,6-$(OH)_2$	X	4·0	250 (4·01); *273* (*3·96*); 356 (3·74)
	X^{--}	12	254 (4·22); *280* (*3·91*); 368 (3·86)
	Y	4·0	270 (4·02); 298 (3·82)
	Y^{--}	12	270 (3·98); *287* (*3·86*)
4,6-$(OH)_2$-2-NH_2 ...	X	4·0	276 (4·16); 388 (3·42)
	X^{--}	0·1M-NaOH)	255 (4·28); *275* (*4·07*); 394 (3·86)
	Y	4·0	277 (4·17); *300* (*4·09*)
	Y^{--}	(0·1M-NaOH)	276 (4·19)

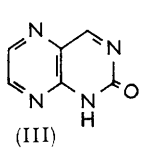
C=N double bond which, if the carbon has a sufficiently large net positive charge to facilitate attack by nucleophilic reagents, will undergo addition of water. This effect is further enhanced by cation formation if the proton adds to a nitrogen atom of the same ring. For example, in acid solutions pteridine [8] and quinazoline [13,14] are rapidly converted into the cations of the corresponding 3,4-dihydro-4-hydroxy-derivatives. In the anions, the formal negative charge would be expected to reside on the oxygen rather than on a ring-nitrogen atom. This leads to double-bond formation between carbon-2 and nitrogen-1 in 2-hydroxypteridine, and between carbon-6 and nitrogen-5 in 6-hydroxypteridine. In both cases, loss of the bound water molecule would restore aromatic character, so that the anhydrous form of the anion is stabilised.

The differences in free energies (ΔG) between hydrated and anhydrous neutral molecules is not very great—the highest ratio of [Y]/[X] (400 for 2,6-dihydroxypteridine) corresponds only to $\Delta G = 3{\cdot}6$ kcal. $mole^{-1}$—so that whether or not hydration occurs depends on a rather sensitive interrelation of a number of factors. That 2- but not 4-hydroxypteridine adds water can be explained by the two effects which operate in the former, but are absent from the latter, case. Thus, the electron density on carbon-4 in 2-hydroxypteridine would be reduced by the conjugation with the carbonyl group in O=C–N=C–; it would also be reduced because of the linkage to the π-electron-deficient pyrazine ring. An

[13] Albert, Armarego, and Spinner, *J.*, 1961, 5267.
[14] Armarego, *J.*, 1962, 561.

alternative explanation is that the hydrated form of 2-hydroxypteridine is stabilised by a urea-type resonance which cannot occur in 4-hydroxypteridine.[9] Discrimination between 6- and 7-hydroxypteridines on similar grounds is more difficult, and it has been suggested [6] that the hydrated form of 6-hydroxypteridine owes its stability to a 4-aminopyridine type of resonance stabilisation (V); similar structures cannot be written for 7-hydroxypteridine so as to locate a positive charge on nitrogen-5.

This suggestion could also explain the failure of 2,7-dihydroxypteridine to add water across nitrogen-3 and carbon-4 because the double-bond character of the bond between these atoms would be diminished by a similar resonance stabilisation involving the amide form of anhydrous 7-hydroxypteridine. That some transannular effect is important is further indicated by comparing results for 6-hydroxy- and 4,6-dihydroxy-pteridine: the 4-hydroxy-group reduces by a factor of 100 the extent of hydration across the 7,8-double bond.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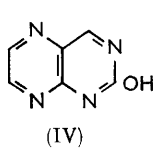
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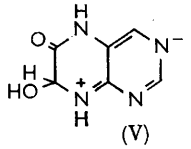
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Xanthopterin.—The consistent trends in pK values, equilibrium constants, and absorption maxima, as set out in Tables 1 and 2, lead to the conclusion that xanthopterin undergoes reversible hydration across the 7,8-double bond to give the species (I) and (II). Further, their spectra and other properties enable Schou's [2] fluorescent "enol" to be identified as xanthopterin (I) and his non-fluorescent "keto" form as the covalent hydrate (II). The ultraviolet spectrum of the latter is similar to that of 7,8-dihydroxanthopterin [$\lambda_{max.}$ 268 mμ, 300 mμ (inflection); log ε 4·10, 3·85 [15]], from which it differs only by having an alcoholic hydroxyl group in place of a hydrogen atom. We confirm Schou's observations that species (I) and (II) are present in almost equal amounts in neutral solutions at room temperature and that the first acid dissociation constant of xanthopterin is about 6·5. However, the pH-dependence of the properties of equilibrated xanthopterin solutions is more complicated than Schou suggested, because of the simultaneous operation of four different pK's, namely, two each for the forms (I) and (II), and the equilibria among the neutral species and mono- and di-anions.

As with the simpler hydroxypteridines, water-addition to xanthopterin, and its removal, are acid–base-catalysed reactions. Log k_1 for the acid-catalysed hydration of xanthopterin at 20° is 2·77, and is comparable with the values of 2·23 for 4,6-dihydroxypteridine and 3·20 for 6-hydroxypteridine.

Oxidation of Hydroxypteridines by Xanthine Oxidase.—Bergmann and Kwietny [10] studied the rates of oxidation of a number of hydroxypteridines in buffers of pH 8·0 at 28° in the presence of xanthine oxidase but were unable to find any correlation between structure and reactivity. Their findings have now been re-examined in view of the importance of covalent hydration in some of the substrates used. Schou's results show that xanthopterin is readily oxidised to leucopterin if xanthine oxidase is present, whereas the corresponding "hydrated" species is not attacked. Atomic (Leybold) models suggest a possible steric explanation for this difference: "anhydrous" hydroxypteridines, including xanthopterin, are planar molecules, irrespective of whether they exist in amide or enol form, and hence might be adsorbed by van der Waals forces on to a flat portion of the xanthine oxidase molecule. This planarity is lost in the "hydrated" forms of pteridine and hydroxypteridines, so that such intimate contact would no longer be possible, with consequent loss of reactivity.

If this explanation is correct, the figures in Table 1 should enable some predictions to be made concerning the readiness of enzymic oxidation of the various pteridine derivatives.

[15] Hitchings and Elion, *J. Amer. Chem. Soc.*, 1949, **71**, 467.

TABLE 3.

Hydration and relative rates of oxidation of hydroxy- and dihydroxy-pteridines.

Reaction rates are from ref. 10, with xanthine oxidase; xanthine is taken as standard = 100. Values in parentheses are approximate.

Hydroxypteridine	2-		4-	6-		7-
% hydrated in neutral molecule	99·7		0	99·2		0
Initial oxidation rate	0·9		53	0		37
Dihydroxypteridine	2,4-	2,6-	2,7-	4,6-	4,7-	6,7-
% hydrated in neutral molecule	0	99·75	0	55	0	0
Initial oxidation rate	21	0	21	(7)	38	(7)

At pH 8, hydration–dehydration is rather slow in solution so that the initial oxidation rates should depend on the equilibrium concentration of each " anhydrous " species present. Hence the mono- and di-hydroxypteridines that exist in solution mainly in the " hydrated " form should be least rapidly oxidised by enzyme action. (By contrast, the hydrated forms are much more readily oxidised than are true hydroxypteridines by chemical reagents.[5]) Table 3 shows this to be so. It is not at present known whether the large variations in the rates of oxidation of substituted purines may be due in part to a similar effect.

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