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OXIDATION OF METHYL DERIVATIVES OF PTERIDIN-4-ONE, LUMAZINE AND RELATED PTERIDINES BY BOVINE MILK XANTHINE OXIDASE

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Summary

1. Pteridin-4-ones, methylated at nitrogen or carbon, *N*-methylated lumazines and related oxopteridines were studied as substrates of a highly purified bovine milk xanthine oxidase (xanthine : oxygen oxidoreductase, EC 1.2.3.2).

2. The enzyme can oxidise at high rates both uncharged and anionic substrates. Variation of enzymic activity with pH is mainly due to pH-dependent changes in the active enzymic center.

3. Milk xanthine oxidases at different stages of purification convert pteridin-4-one into the 4,7-dione (compound 13 in this article).

4. Methylation at C-6 in the pyrazine moiety enhances enzymic attack at C-2 in the pyrimidine ring. *N*-Methylation may increase or reduce rates of oxidation.

5. For oxidation at C-2, the most favorable form of the substrate bears a double bond at C(2) = N(3). Attack at C-7 is enhanced strongly in structures bearing a double bond at C(6) = C(7).

6. In general, pteridines react with xanthine oxidase as non-hydrated molecules. However, oxidation of 8-methyllumazine at C-7 may take place by dehydrogenation of the 7-CHOH group of the covalently hydrated molecule.

Introduction

Studies on the characteristic structural features, which enable hypoxanthines, xanthines and purine-6,8-diones to serve as substrates of milk xanthine oxidase (xanthine : oxygen oxidoreductase EC 1.2.3.2), have revealed that certain groups of the form HN-C=N are needed to bind these purines to the enzyme [1]. It is still unknown which portion of the active enzymic center is involved in the association with such groups.

Several questions remained so far without a satisfactory solution. If a substrate is present as a mixture of neutral molecules, containing NH groups, and of the corresponding anions, which of these forms is attacked by xanthine oxidase? If a substrate lacks NH- or HN-C=N groups, can it be oxidised by this enzyme?

Experiments with pteridines may contribute to the solution of these problems. We shall show in the present study that the enzyme rapidly attacks several pteridines which cannot form anions at all, as well as others which are present nearly 100% as anions.

Yen and Glassman [2] have concluded that the same active center in milk xanthine oxidase is responsible for oxidation both of purines and pteridines. This claim was based on the observation that 8-azaguanine showed similar inhibitory effects on the oxidation of hypoxanthine and of pterine. Therefore we may assume that results with purines and pteridines as substrates of xanthine oxidase are comparable and thus can form the basis for a unified interpretation of the mode of action of the enzyme.

Materials and Methods

All pteridines used in this study, either as substrates or for identification of the products of enzymic oxidation, are recorded in Table I. Paper chromatography, paper electrophoresis and ultraviolet spectroscopy served as methods of identification.

p*K* values were determined from plots of λ_{max} as function of pH. The following buffers were used: pH 5, McIlvaine's citric acid-phosphate buffer; pH 6—7, 0.067 M Sørenson's phosphate buffer; pH 8, 0.02 M Sørenson's phosphate buffer; pH 9—10, Clark and Lub's 0.1 M borate buffer; pH 11, Sørenson's 0.1 M glycine II buffer.

NMR spectra were measured on a JEOL MH-100 instrument, using TSP (sodium 3-trimethylsilyl[2,2,3,3-d_4]propionate) as internal standard. All reactions and analytical procedures were carried out in the dark, because of the photosensitivity of many pteridines.

High-voltage electrophoresis

For this analytical procedure, a Shandon No. 2550 apparatus, with a 2542 power supply of 10 000 V d.c., was used. The oxidation mixture was brought to dryness and extracted with warm Me_2SO. The extract was spotted on Whatman paper No. 1. After completion of the electrophoresis, the paper strip was dried and the spots identified by their fluorescence under ultraviolet light (see Table II).

Bovine milk xanthine oxidase

Two enzyme preparations were used:

(1) A highly purified xanthine oxidase (30 000 units/ml; 115 mg of protein/ml) was a gift of Professor R.C. Bray, Dept. of Biochemistry, University of Sussex, England [15]; ratio of activity/E_{450} at 25°C $\approx$ 120.

(2) The commercial milk xanthine oxidase of Sigma Co., Saint Louis, Missouri, U.S.A., had 2000 units/ml (55.2 mg protein/ml) and a ratio of activity/

TABLE I

LIST OF PTERIDINES REQUIRED FOR THE ENZYMIC EXPERIMENTS

No.	Methyl groups at positions	Source [a]	Reference
Pteridin-4-ones			
1	—	D.J.B. [b]	3
2	3	W.P. [c]	3
3	1	Z.N. [d]	3
4	7	D.J.B.	4
5	6,7	Z.N.	5
6	3,6,7	Z.N.	5
7	1,6,7	Z.N.	5
Pteridine-2,4-diones (lumazines)			
8	—	W.P.	6
9	3 [e]	W.P.	7
10	1	W.P.	6
11	1,3	W.P.	7
12	7	W.P.	8
21	6,7	W.P.	6
22	1,6,7	W.P.	6
30	8	W.P.	9
Pteridine-4,7-diones			
13	—	W.P.	4
14	3	W.P.	4
Pteridine-2,4,6-triones			
15	—	Z.N.	10
16	3		11
17	1		11
18	1,3		11
Pteridine-4,6,7-triones			
19	—	D.J.B.	12
20	1	(new compound) [f]	
Pteridine-2,4,7-triones			
23	—	W.P.	13
24	3	W.P.	13
25	1	D.J.B.	13
26	1,3	W.P.	13
31	8	N.W.J. [g]	13
Pteridine-2,4,6,7-tetraones			
27	—	D.J.B.	10
28	1		14
29	3 [e]		14

[a] If no source is given, the compound was synthesised in the present study.

[b] D.J.B.: gifts from Professor D.J. Brown, Department of Medicinal Chemistry, Canberra, Australia.

[c] W.P.: gifts from Professor W. Pfleiderer, University of Konstantz, West Germany.

[d] Z.N.: synthesised by Dr. Zohar Neiman of this department.

[e] The known compounds 9 and 29 were synthesised by a new method, to be published separately.

[f] Synthesis of this still unknown pteridine will be described separately.

[g] N.W.J.: gift from Professor N.W. Jacobsen, University of Queensland, St. Lucia, Brisbane, Australia.

TABLE II

ANALYSIS OF OXIDATION MIXTURES

No.	Compound	R_f in solvent	Fluorescence	Mobility (cm/h per 1000 V)		
				pH 5	pH 7.5	pH 10
Oxidation of pteridin-4-one, compound 1 [b]						
1	Pteridin-4-one	0.53	purple		1.9	
8	Pteridine-2,4-dione	0.37	yellow		0.8	
13	Pteridine-4,7-dione	0.20	violet		5.7	
23	Pteridine-2,4-7-trione	0.23	violet		4.6	
Oxidation of 3-methylpteridin-4-one, compound 2 [c]						
		B				
2	3-methylpteridin-4-one	0.48	purple			−1.5
9	3-methylpteridine-2,4-dione	0.58	green-yellow			+6.7
14	3-methylpteridine-4,7-dione	0.08	purple	−0.2		
24	3-methylpteridine-2,4,7-trione	0.17	sky blue	+4.7		

[a] The synthetic mixture containing starting material, all possible intermediates and the end-product of enzymic oxidation, was brought to dryness in vacuo and the residue extracted with warm Me_2SO. This solution was spotted on Whatman paper No. 1. Spots were visualised in ultraviolet light of 366-nm wavelength.

[b] Paper chromatography in solvent A (95% ethanol/12.5% ammonia, 4 : 1, v/v) separated three spots, corresponding to compounds 1 and 8 and a mixture of 13 and 23. The last spot was again extracted with Me_2SO and the material spotted on Whatman paper No. 1. High-voltage electrophoresis in Sørenson's buffer of pH 7.5 was carried out for 4 h. Since the spots were very compact, the results of separation are unequivocal. This is critical for identification of the only intermediate in the oxidation of pteridin-4-one as the 4,7-dione 13.

[c] Paper chromatography in solvent B (isopropanol/dimethylformamide/water, 13 : 5 : 2, v/v) separated two spots. One comprised a mixture of compounds 2 and 9, and the second of the derivatives 14 and 24. The first spot was reextracted and subjected to paper electrophoresis in Clark and Lub's borate buffer, pH 10. This separation was used to prove that the lumazine 9 was absent during the conversion of 2 to 24. The second spot was subjected to electrophoresis in McIlvaine's citrate-phosphate buffer of pH 5 to identify both the exclusive intermediate 14 and the end-product 24.

$E_{450} \approx 60$ at 25°C. This enzyme exhibited essentially the same specificity for pteridine substrates as the preparation of Dr. Bray.

Definition of enzyme unit: At pH 8 and 29°C, 1 unit of xanthine oxidase produces 1.5 μg/ml of uric acid, using 5×10^{-5} M xanthine as substrate.

Catalase (Worthington) had an activity of 50 000 units/ml; 3 μg/ml protein. At a concentration of 0.06 M H_2O_2, 1 unit of catalase decomposes 1 μmol of H_2O_2/min at 25°C and pH 7. In all enzymic assays, the final concentration of catalase was 10 units/ml.

Determination of enzymic rates

All components were dissolved in buffer of pH 8. Substrate and catalase were incubated together for 3 min and the reaction was started by adding xanthine oxidase. All values in the tables refer to final concentrations of the components; total volume was 3 ml. The solutions were placed into the thermospacer of a Cary 14 ultraviolet spectrophotometer, kept at 29°C. Readings were taken at the wavelengths specified in Tables V—VII.

Fast substrates were used at their optimal concentrations, as determined

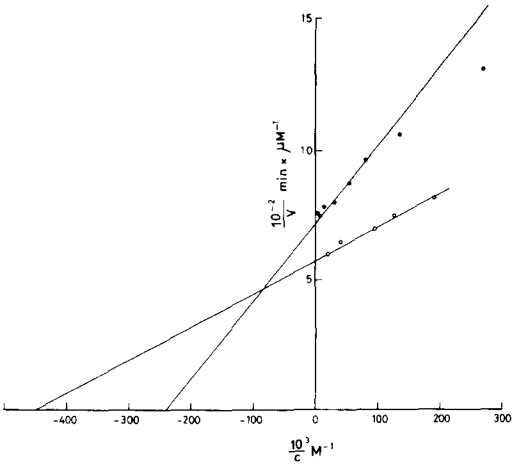


Fig. 1. Lineweaver-Burk plots for enzymic oxidation of 3-methylpteridine-2,4-dione, compound 9 (●———●, enzyme 10 units/ml) and pteridine-4,7-dione, compound 13 (○———○, enzyme 4 units/ml), at pH 8, 29°C.

from plots of rates versus log (substrate concentration). Substrates reacting at a relative rate of 1% or less, were employed at a standard concentration of 5×10^{-5} M, since their pS optimum could not be measured exactly.

Enzymatic rates were derived from the initial linear portion of the curves, showing ΔA as function of time. Relative rates were calculated by assuming a linear relation between enzyme concentration and rate of oxidation for all substrates. The value 100 was assigned to the rate of xanthine at its pS optimum with any given enzyme concentration. It is to be understood that relative rates below 1% cannot be determined accurately.

V and K_m values were obtained from Lineweaver-Burk plots (Fig. 1); straight lines were fitted by least squares (see Table III).

Results

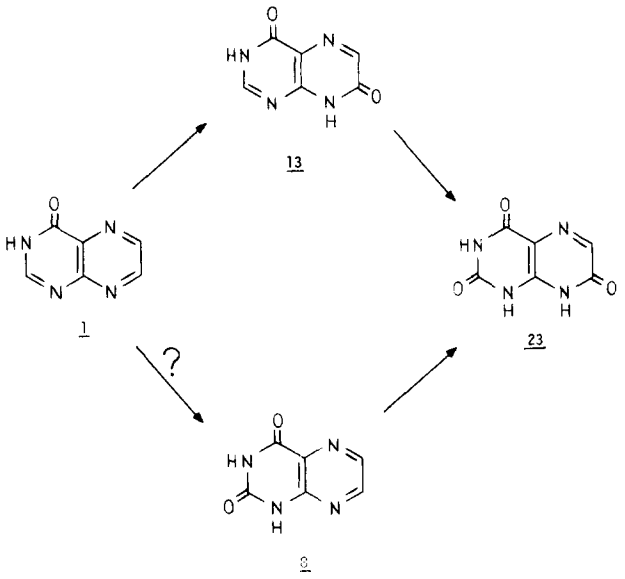
(1) Pathway of oxidation of pteridin-4-one, compound 1

Some time ago we reported that pteridin-4-one (compound 1) is oxidized via the 4,7-dioxo intermediate 13 to pteridine-2,4,7-trione 23 (Scheme 1) [16]. However, Forrest et al. [17] claimed that the only intermediate in this reaction is lumazine 8. We have re-investigated this case with the new, highly purified enzyme of Bray as well as with a commercial milk xanthine oxidase and confirm our original statement for either preparation. We do not know the reason for this discrepancy.

(2) pS- and pH-optima

For fast-reacting oxopteridines, we have determined enzymic rates as function of substrate concentration. Some representative figures are given in Table III. The pS optima vary over about a 60-fold range; the pS vs. activity curves are of two types, exhibiting either sharp peaks or broad maxima, similar to our previous findings with purines [1].

Scheme 1.



For compounds 1, 7, 8 and 13, the pH optimum is near 8 (Fig. 2). In the case of 7, which cannot form an anion, the pH dependence of the reaction evidently expresses changes in the active center of the enzyme. Here activity declines much faster on the alkaline than on the acid side. This may be due for instance to loss of the charge from an $-NH_3^+$ ion, releasing an anionic group in or near the active center so that it can now approach one of the metal atoms to form an inactive complex. On the other hand, compound 13 is present at pH 8 about 97% and at pH 9 about 76% as monoanion. Therefore 13 is capable of

TABLE III

KINETIC CONSTANTS FOR OXIDATION OF PTERIDINES BY MILK XANTHINE OXIDASE

No.	Compound	V [a] (%) (± S.D.)	s_{opt} [a] × 10^5 (M)	V [b] (%)	K_m [b] × 10^6 (M)
1	Pteridin-4-one	38 ± 8	45 [c]	24	5.6
5	6,7-Dimethylpteridin-4-one	>43	>14 [d]	52	38.5
7	1,6,7-Trimethylpteridin-4-one	>66.5	>32 [d]	78	125
8	Lumazine	19.5	0.7	22.8	2.0
9	3-Methylpteridine-2,4-dione	83	12.5	84	4.25
13	Pteridine-4,7-dione	77 ± 2	8	63	2.0
15	Pteridine-2,4,6-trione	5.2 ± 1.5	1	8.5	3.4
19	Pteridine-4,6,7-trione	43	4	71	7.9

[a] V = relative maximal rate at S_{opt}, from a plot of rates versus log(substrate concentration); S.D. = standard deviation. This figure is given only if more than 2 determinations were carried out. V of xanthine = 100.

[b] From Lineweaver-Burk plots.

[c] The broad maximum of the curve, showing rate as function of log(substrate concentration), permits only approximate evaluation of this figure.

[d] Because of limitations in solubility, these values could only be approached, but were not determined accurately.

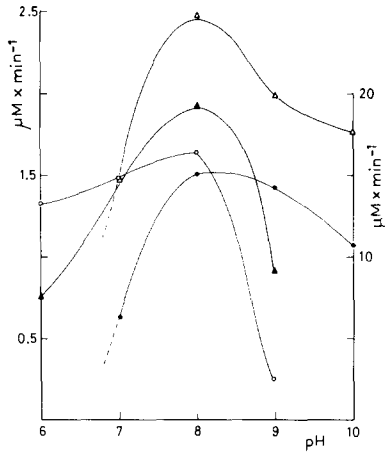


Fig. 2. pH dependence of the oxidation of representative pteridines by milk xanthine oxidase. ▲———▲, Pteridin-4-one, compound 1, $4.3 \cdot 10^{-5}$ M; ○———○, 1,6,7-trimethylpteridin-4-one, compound 7, $5.9 \cdot 10^{-5}$ M; △———△, lumazine, compound 8, $4.5 \cdot 10^{-5}$ M; ●———●, pteridine-4,7-dione, compound 13, $5.2 \cdot 10^{-5}$ M. For all substrates, the final concentration of xanthine oxidase was 4 units/ml, except for compound 7, for which 2 units/ml were used. Right ordinate applies only for compound 13. The rates of 1, 8 and 13 were determined at fixed wavelengths (288, 328 and 328 nm, respectively) throughout the whole pH range, taking into account the change of optical density as function of pH. Compound 7 cannot form an anion.

competing with the anionic group in the enzymic center and thereby suppressing to a large degree the adverse affect of alkalinisation, described above (see Fig. 2). In contrast on the acid side of the curve (below pH 8), the anionic group in the enzymic center loses its charge and now the $-NH_3^+$ ion is free to interact with the anionic group of the substrate 13 and thus to prevent the latter to complex with the active center. At pH 8, compounds 1 and 8 form mixtures of uncharged molecules and anions and thus may exhibit features of either type of pH vs. activity curves (Fig. 2). Since no detailed information is available about the structure of the active center of xanthine oxidase, these considerations remain at present speculative.

NMR spectra of pteridines

Inoue and Perrin [18] have claimed that hydration of pteridines destroys their planar structure and thus interferes with attack by xanthine oxidase. Covalent hydration is especially important in pteridines bearing 2- or 6-oxo-groups [19—21]. We have therefore measured the NMR spectrum of all *N*-methylpteridines, used in the present experiments, provided they were sufficiently soluble in water. Albert et al. [22] have observed that the signals of aromatic protons in the pteridine ring are shifted upfield by 3—4 ppm, when the double bond involved binds water covalently. The data in Table IV show that all pteridines that could be measured, exhibited δ values for the aromatic protons in the range 8—9.5 ppm. Thus substantial spontaneous hydration in aqueous solutions, under the experimental conditions used, is excluded for all compounds tested in Table IV.

(4) Enzymic oxidation of pteridin-4-ones

The similarity of the ultraviolet spectra of pteridin-4-one 1 and its 3-methyl

TABLE IV

PHYSICAL PROPERTIES OF OXOPTERIDINES AND SOME OF THEIR *N*- AND *C*-METHYL DERIVATIVES

No.	Methyl groups at position	λ_{max}(nm)		pK for anion formation	δ(ppm) [b]									
		N[a]	A[a]		2-H		6-H		7-H		N-Me		C-Me	
					N	A	N	A	N	A	N	A	N	A
Pteridine-4-ones														
1	—	310 265 230	333 242	7.9	8.58	8.57	9.00(d) [c]	8.85(d)	9.16(d) [c]	8.89(d)				
2	3	310	—	—	8.71	—	9.02(d)	—	9.15(d)	—	3.75	—		
3	1	322 259 sh 230	—	— —	8.81	—	9.07(d)	—	9.15(d)	—	4.02	—		
4	7	310 270 231	330 242	8.2	8.58	8.49	8.89	8.67	—	—	—	—	2.81	2.76
5	6,7	316 270 237	335 246	9.5	8.58	8.58							2.80 2.83	2.73 [d]
6	3,6,7	315 285 sh 240	—	—	8.64	—					3.78	—	2.79 2.80	—
7	1,6,7	328 237	—	—	8.76	—					4.05	—	2.81 2.86	—
Pteridine-2,4-diones (lumazines)														
8	—	324 243 sh 230	347 270 238	8.1	—	—	8.90(d)	8.76(d)	8.49(d)	8.41(d)				
9	3	326 230	364 269 242	7.7										
10	1	329 230	336 286 240	8.4			8.80(d)	8.81(d)	9.03(d)	8.96(d)	3.73	3.64		
11	1,3	334 238	—	—			8.89(d) [e]	—	9.05(d)	—	3.75(1) [f] 3.53(3)	—		
12	7	325 222 sh	345 273	8.2			8.68	8.37					2.73	2.64

Pteridine-4,7-diones												
13	—	330 287 215	329 297 227	6.5 9.5	8.47 [e]	8.22	8.30	7.88				
14	3	329 288 244 sh	327 292 281 228	6.0	8.62	8.48	8.32	8.08			3.70	3.75
Pteridine-2,4,6-triones												
15	—	369 251 226	386 264 226	5.2 9.3					8.54	8.22		
16	3	372 249 222	380 261 226	5.0 9.5					8.33 [e]	8.05	3.35	3.31
17	1	368 255 217 sh	383 267 221	5.4 9.5					8.53	8.13	3.63	3.54
18	1,3	373 256 227	385 265	4.9					8.60 [e]	8.49 [g] 8.39	3.71(1) [f] 3.49(3)	
Pteridine-4,6,7-triones												
19	—	298 240 216	346 sh 330 318 240	6.7 9.5	— [h]	8.04 [e]						
20	1	355 336 322 310 sh	356 337 322 310 sh	6.5 11.5	8.47	8.36					3.88	3.85

sh: shoulder.

[a] N: neutral molecule; A: anion.

[b] In ^2H_2O, with the appropriate buffer, unless otherwise stated.

[c] d: doublet, due to coupling between 6-H and 7-H.

[d] Only one C-Me signal was observed in the anion. The disappearance of the second signal will be dealt with separately.

[e] In d_6-Me_2SO-^2H_2O, 9 : 1, v/v.

[f] The signal at higher field is assigned to the 3-Me group, which is located between two carbonyls.

[g] In the anion, the 7-H signal appeared as two peaks.

[h] The signals of the neutral form could not be measured because of solubility limitations.

derivative 2 indicates that 1 is present in aqueous solution predominantly as the 3-NH tautomer. Both compounds are attacked at position 7, but the rate for compound 1 is about 300 times higher (Table V). Since the 3-methyl substituent presumably has no steric influence on a reaction proceeding at the remote 7-position, two explanations may be advanced for this large difference in rates:

(a) Compound 1, pK = 7.9, forms at pH 8 about 56% anions (1c—e in Scheme 2), while 2 does not possess a dissociable -NH group. The lack of anion formation may explain the low reactivity of the latter.

(b) The 3-NH group enables the molecule of 1 to assume tautomeric structures (1a, b) with high affinity to the active center. This is impossible with compound 2.

Scheme 2.

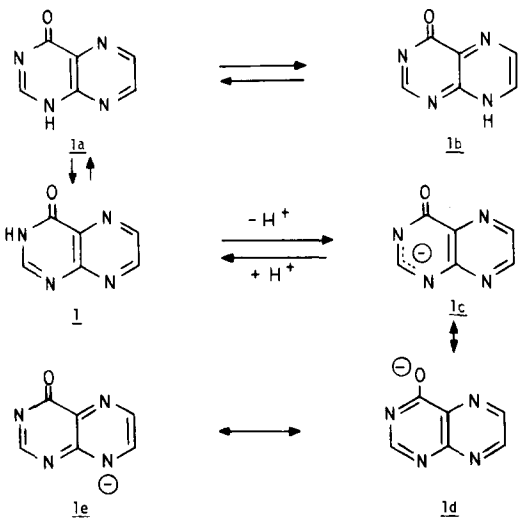


TABLE V

ENZYMIC OXIDATION OF PTERIDIN-4-ONES

All experiments at pH 8, 29°C.

No.	Methyl groups at positions	Wavelengths used for rate measurements (nm)	Xanthine oxidase final concn. (units/ml)	Position attacked	V * (%) ± S.D.	Number of experiments
1	—	288; 258	2	7	38 ± 8	9
2	3	340; 330	20	7	0.1 ± 0.025	8
3	1	280	2	2	10.3 ± 0.7	3
4	7	370; 310; 270	3.3	2	11.3 ± 1.5	4
5	6,7	335	2	2	>43 **	3
6	3,6,7	w.s. ***	10	—	—	2
7	1,6,7	255	2	2	>66.5 **	3

* Rate at pS optimum, divided by rate of xanthine at same enzyme concentration. Only compounds 2 and 6 were used at $5 \cdot 10^{-5}$ M final concentration. S.D. = standard deviation.

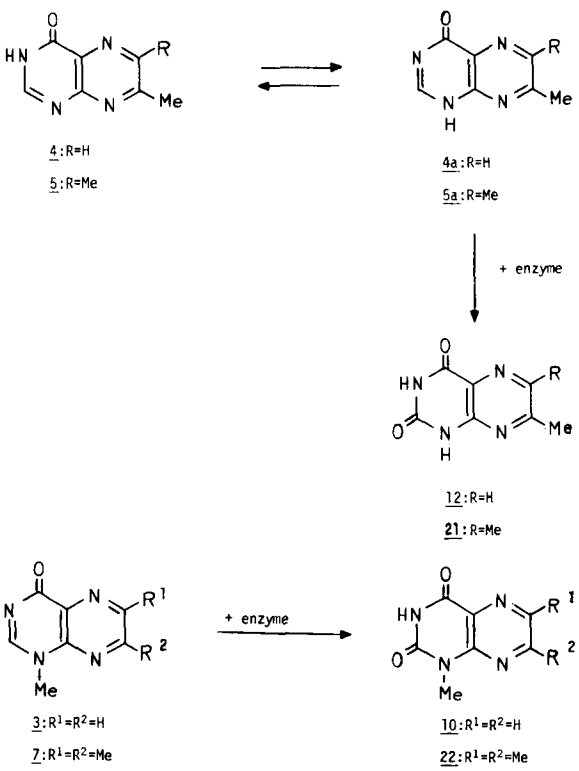
** With these substrates the maximal rate could only be approached, because of solubility limitations. The maximal concentrations used were: for compound 5, $1.4 \cdot 10^{-4}$ M, and for compound 7, $3.2 \cdot 10^{-4}$ M.

*** w.s.: whole spectrum.

The first possibility may be rejected because of the high rate of oxidation of the 1-methyl derivatives 3 and 7, which also cannot form anions. These compounds give clear-cut evidence that xanthine oxidase can attack uncharged substrates. Therefore we may assume that 1 reacts with the enzyme in the tautomeric form 1b (Scheme 2).

The 7-methyl derivative 4 is oxidised at C-2, probably as tautomer 4a (Scheme 3), which corresponds to 1a and 3. Clearly, attack at C-2 requires placement of a double bond at position 2-3, either by methylation at N-1 (as in 3 and 7, Scheme 3) or by tautomerisation (as in 1a and 4a).

Scheme 3.



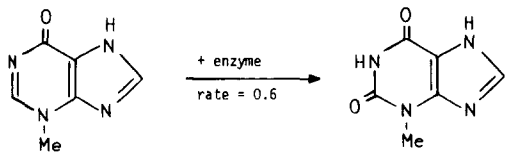
In this connection it should be realised that an active structure may also result from mesomerism in the anion of 1 (1c—e in Scheme 2). Likewise in the anion of 4, a double bond can be placed at position 2—3.

The 6,7-dimethyl derivative 5 reacts about 5 times faster than the corresponding 7-methyl derivative 4; 3,6,7-trimethylpteridin-4-one 6 is refractory. The rate enhancement for 5 may be ascribed to the inductive effect of the 6-methyl substituent, which increases polarisation of the 5-6 double bond and thereby also the nucleophilicity of N-5. Under this hypothesis, 4-C=O and N-5 together are responsible for complexing of these substrates with a certain group in the enzymic center.

However, another possibility should also be considered: A 6-methyl substituent may sterically prevent an unfavorable alternative type of complex formation (see Discussion).

The relatively high rate of 3 is in marked contrast to the low rate of oxidation (0.6) of 3-methylhypoxanthine at C-2 [1]. The latter compound exists in solution as 7-NH tautomer [23] (see Scheme 4); in this detail its structure differs from that of 3.

Scheme 4.



(5) Pteridine-2,4-diones as substrates of xanthine oxidase (Table VI)

All members of this series are oxidised at position 7, C-6 being resistant to enzymic attack [24]. Thus the 7-methyl derivative 12 is refractory. Comparison of the pK values of 8, 9 and 10 and the long-wave ultraviolet maxima of their anions (Table IV) suggests that in the mother substance 8 both 1- and 3-NH participate in monoanion formation, while Pfleiderer [7] has claimed the ionisation sequence 1-NH → 3-NH for mono- and dianion formation.

Scheme 5.

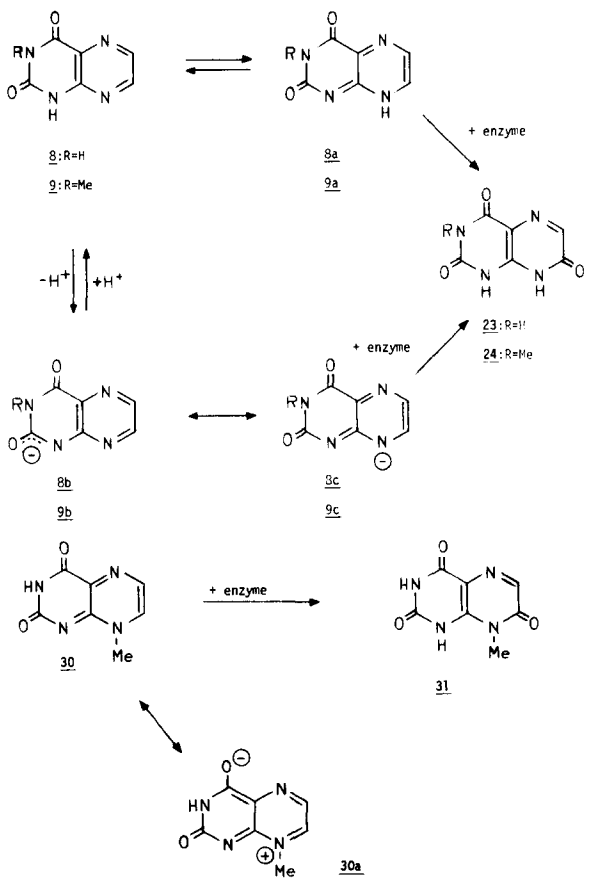


TABLE VI

ENZYMATIC OXIDATION OF PTERIDINE-2,4-DIONES

Conditions as in Table V. All members of this group, which serve as substrates, are attacked at position 7.

No.	Methyl groups at positions	Wavelengths used for rate measurements (nm)	Xanthine oxidase, units/ml, final concn.	V * (%) ± S.D.	Number of experiments
8	—	328	4	19.5 ± 5	4
9	3	328	2	83 ± 13	3
10	1	270	4	approx. 0.13	3
11	1,3	300	6.7	approx. 0.01	2
12	7	w.s. *	3.3	—	2
30	8	400 350	2	23	2

* w.s.: whole spectrum.

The rate of oxidation of 9 to 24 is about 4 times higher than that of the conversion 8 → 23. Scheme 5 indicates that 9 can bind to the enzyme either via the group HN(1)-C-N(8) (9) or via N(1) = C-N(8)H (9a). The latter resembles tautomer 1b in Scheme 2. The -NH group involved dissociates at pH 8 to 67% to give the mesomeric pair of anions 9b,c. Resonance form 9c resembles 1e in Scheme 2. Both 9a and 9c may explain rapid attack at position 7. In contrast,

TABLE VII

OXIDATION OF DI- AND TRIOXOPTERIDINES BY XANTHINE OXIDASE

Conditions as in Table V.

No.	Methyl groups at positions	Wavelengths used for rate measurements (nm)	Xanthine oxidase, units/ml, final concn.	Position attacked	V * (%) ± S.D.	Number of experiments
Pteridine-4,7-diones						
13	—	328	1	2	77 ± 2	3
14	3	240 340	10	2	0.25 ± 0.04	4
Pteridine-2,4,6-triones						
15	—	300	10	7	5.2 ± 1.5	2
16	3	320 340 350 380	6.7	7	1 ± 0.3	2
17	1	w.s. **	10	7	very slow	2
18	1,3	w.s.	10	—	—	2
Pteridine-4,6,7-triones						
19	—	360	0.67	2	43	2
20	1	345	20	2	approx. 0.15	2

* Rate at pS optimum, divided by rate of xanthine at its pS optimum and at the same enzyme concentration. S.D. = standard deviation.

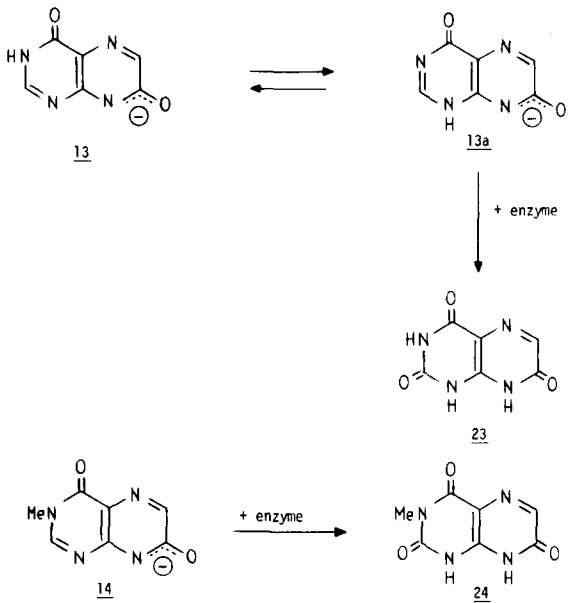
** w.s.: whole spectrum.

in 8 either in the neutral molecule or its monoanion additional modes of complexation are possible, e.g. via HN(3)-C(4)O or HN(3)-C(2)O. These are less favorable for oxidation at C-7, as evidenced by the low rate of 10. In the latter, these groupings can be utilised, but N-1 cannot participate in binding; the rate decreases approx. 150-fold, as compared to 8, or 650 times relative to 9 (Table VI). If both positions 1 and 3 are methylated, as in 11, the enzymic rate is reduced further to the limit of measurable values.

(6) Enzymic oxidation of other di- and trioxopteridines (Table VII)

The 4,7-dioxo derivatives 13 and 14 are present at pH 8, to 97 and 99%, respectively, as anions. From their pK values and their ultraviolet spectra (Table IV) it is concluded that in the monoanion of 13 the 8-NH group is ionised [4]. Therefore we may depict the tautomeric forms of 13 as shown in Scheme 6.

Scheme 6.



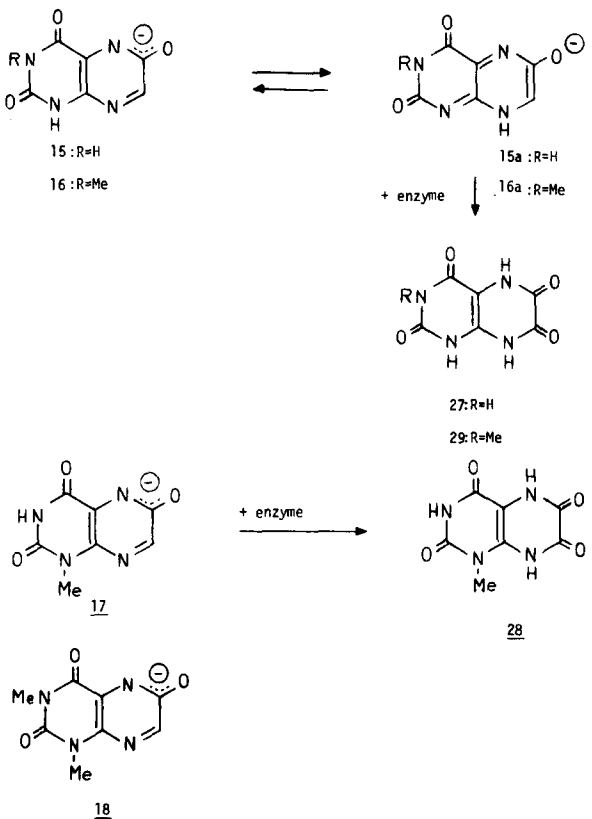
Tautomer 13 a is assumed to represent the active for enzymic oxidation at C-2, to yield 23.

The rate of 13 is about 300 times higher than that of its 3-methyl derivative 14, but in spite of the inability of 14 to tautomerise in the pyrimidine moiety, it is converted to 24 (see Scheme 6) at a measurable rate. In fact the ratio of rates 13/14 is very similar to the ratio 1/2.

In the series of pteridine-2,4,6-triones, the mother substance 15 is oxidised about 5 times faster then its 3-methyl derivative 16, while attack of the 1-methyl isomer 17 is extremely slow (Table VII). The progress of the oxidation 17 → 28 (see Scheme 7) could be demonstrated only by chromatographic analysis.

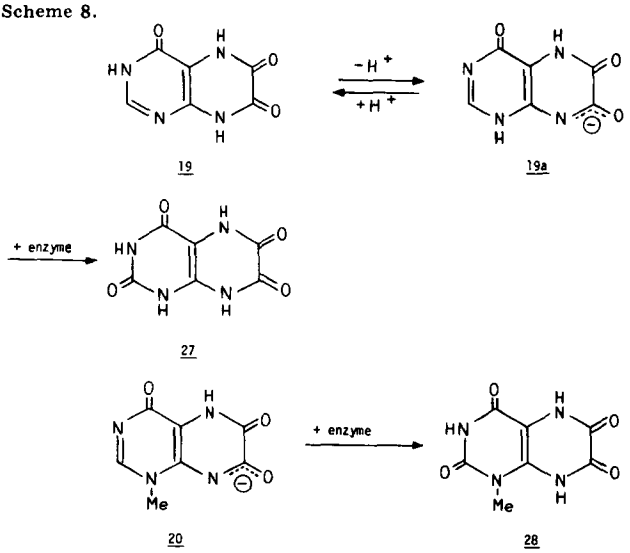
The pK of 18 and the ultraviolet spectrum of its anion are similar to the dissociation constants for monoanion formation in compounds 15—17 and to the λ_{max} of their anions. Thus the first dissociation step in this series of pteridines involves 5-NH [11]. Scheme 7 may explain why 17 is oxidised at such a low

Scheme 7.



rate and why 18 is refractory, both substrates being unable to form a tautomer with the N(1) = C—N(8)H grouping (like 15a and 16a), required for attack at C-7.

Scheme 8.



Conversion of pteridine-4,6,7-trione 19 to 27 is interpreted in Scheme 8. Since the ultraviolet spectra of the monoanions of 19 and its 1-methyl derivative 20 are not very different, it appears possible that both these substrates suffer dissociation of an -NH group in the pyrazine ring. In the anion of compound 20, a C = N double bond is fixed in the proper 2,3 position, and by analogy the corresponding structure of 19 may be formed by tautomerisation (Scheme 8). Therefore it is surprising that 20 is oxidised at only 1/280 of the rate of 19, and about 40 times slower than 3.

(7) Can xanthine oxidase attack carbon-carbon double bonds?

For rapid attack at C-2, we have assumed placement of a double bond at C(2) = N(3) (see Schemes 3, 6 and 8). On the other hand, for oxidation at position 7, tautomers or mesomers with a C(6) = C(7) double bond appear to be required (see Schemes 2, 5 and 7). In order to test the latter hypothesis, we have examined 8-methylpteridine-2,4-dione 30, in which the 6,7-double bond appears to be fixed. This derivative is attacked rapidly at C-7 to yield 31 (see Table VI and Scheme 5).

Discussion

Our experiments show that both uncharged molecules and anions can serve as substrates for milk xanthine oxidase. Furthermore, free NH- or HN-C-N groups are not indispensable for substrates that are attacked at high rates; for instance pteridine 7 reacts at a rate approaching that of xanthine. However, NH- groups impart to the molecule great flexibility, such as formation of tautomers or of anions with their various mesomeric structures. Such special forms may represent the reactive state of the substrate.

The results obtained in the present study with pteridines, permit some important comparisons with the behavior of purines towards milk xanthine oxidase. Among all *N*-monomethylated hypoxanthines, only the 3-methyl derivative is attacked by the enzyme (Scheme 4); none of the *N,N'*-dimethyl analogs, including 3,7-dimethylhypoxanthine, serve as substrate [1]. In the series of pteridin-4-ones, the corresponding 1-methyl derivative 3 is rapidly oxidised, at a rate approx. 17 times higher than that of 3-methylhypoxanthine; the reaction of 1.6,7-trimethylpteridin-4-one 7 proceeds as much as 110 times faster. The important structural difference lies in the replacement of the 7-NH-group in these hypoxanthine derivatives by a N(5) = C(6) grouping in the corresponding pteridin-4-ones.

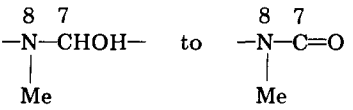
The 4-fold acceleration of the enzymic oxidation of lumazine 8, following methylation at N-3 (9), corresponds to the increased rate of 1-methylxanthine as compared to xanthine [1]. Similarly, the 1-methyl group in 10 reduces the rate of oxidation sharply and the corresponding 3-methylxanthine is completely refractory. Thus in both series, the point of attachment of the alkyl substituent to the uracil moiety plays a decisive role. Since in 9 and 10 the same position is attacked, we conclude that blocking of N-3 by methylation prevents an unfavorable mode of binding involving 3-NH. We may consider the enzyme molecule as a host compound which can complex with a substrate in different ways. The rate of electron- or hydride-transfer [25] apparently depends on the

specific structure of the host-guest complex. Methylation at a suitable position (e.g. N-3 in lumazines or N-1 in xanthines) reduces the total number of modes of complexation and this may increase the probability of formation of the most suitable complex. In contrast, blockade of N-1 in lumazines or of N-3 in xanthines prevents the appropriate mode of binding, shown for instance in Scheme 5.

An analogous explanation may apply to the observation that a 6-methyl substituent enhances enzymic attack at C-2 in pteridin-4-ones (Table I). Altogether it may be concluded that a multiple-point attachment between enzyme and substrate is required to permit rapid reaction.

A special case of great importance is represented by 8-methyllumazine 30. In purines, only C = N groups are available for enzymic oxidation. In the pteridine series, tautomeric structures can be formed containing a C(6) = C(7) double bond. The possibility of oxidation of such a group is suggested by the rapid attack of xanthine oxidase on 30. The following explanations should be considered for this reaction:

(a) The compound reacts as covalent hydrate, in which a hydroxyl group is attached to C-7. Such a hydration process has been demonstrated by Pfleiderer et al. [9] for the anion of 30. However, this pteridine, pK = 10.1, is present at pH 8 to more than 99% as neutral molecule and exhibits identical ultraviolet spectra at pH 6 and 8. Thus the absence of a measurable percentage of hydrated forms under the conditions used here for enzymic oxidation is indicated. Hydration may still take place in the enzyme-substrate complex, if xanthine oxidase indeed could dehydrogenate the group:



In the purine series, no evidence for the formation of hydrated intermediates has been found so far.

(b) The compound can form a zwitterion such as 30a (Scheme 5). If this were the active form of the substrate, the question arises whether xanthine oxidase can attack a cationic group such as -N(Me)$^+$ = CH-. This problem will be discussed in a forthcoming publication.

(C) The enzyme can attack a carbon-carbon double bond that is bordered by one or two tertiary nitrogen atoms. This could be tested by the use of suitable ethylene derivatives.

At present no decision about any of these reaction mechanisms is possible.

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