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OXIDATION OF HYPOXANTHINES, BEARING 8-ARYL OR 8-PYRIDYL SUBSTITUENTS, BY BOVINE MILK XANTHINE OXIDASE

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Summary

1. Hypoxanthines, bearing at position 8 aryl or pyridyl substituents, are converted by bovine milk xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2) into the corresponding xanthines at low rates. Oxidation is accelerated considerably when the 8-pyridyl substituents are quaternised.

2. In the enzymic oxidation of quaternary 8-pyridylhypoxanthines a lag phase precedes the attainment of a constant, maximal reaction rate. It is assumed that the delay is due to a relatively slow conformational change in the active enzymic center.

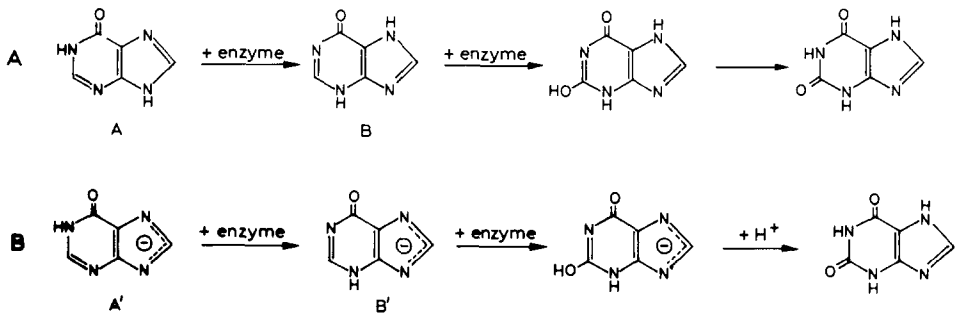
3. In 8-(3′-*N*-methylpyridinio)xanthine betaine, also the pyridinium moiety is attacked at high pH (9—11) to yield an *N*-methyl-2-pyridone. The analogous pyridone is the only oxidation product of 1-methyl-8-(3′-*N*-methylpyridinio)-hypoxanthine betaine, which is not attacked in the pyrimidine ring.

4. The cationic substrates are attracted to the enzyme by an anionic group, which probably forms an ion pair with a protonated amino group in or near the active center.

Introduction

Recently we have studied the conversion of hypoxanthine to xanthine and other, related reactions by bovine milk xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) [1]. Since at the enzymic pH optimum of 8, hypoxanthine (p*K* 8.4) is present as a mixture of neutral molecules and anions, Scheme 1 was proposed for the oxidation of these two forms of the substrate.

An 8-phenyl substituent can exert two opposing effects on the enzymic oxidation of hypoxanthine [2]: (a) The aryl group can increase the affinity by binding to a hydrophobic group in or near the active center of the enzyme. (b) If the conversion of 8-substituted hypoxanthines requires the activation processes of Scheme 1, then a bulky 8-aryl group can hinder tautomerisation in the imidazole ring. Similarly if one of the imidazole nitrogens has to attach itself to

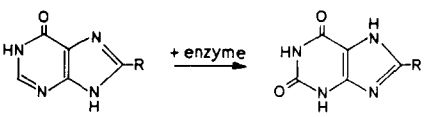


Scheme 1. Enzymic oxidation of hypoxanthine to xanthine, (A) as uncharged molecule, (B) as anion.

an appropriate group in the active center, such binding will be impeded by the bulky 8-phenyl substituent. Consequently, the rate of oxidation should be reduced.

The benzene ring lends itself to a variety of substitutions. Baker et al. [3] have studied a number of 8- and 9-arylhypoxanthines and -adenines as inhibitors of milk xanthine oxidase. Substitution by a *m*-nitrophenyl group produced more effective inhibition of xanthine oxidase than introduction of a *p*-nitrophenyl substituent, but no clear relation with the electronic influence of the substituents in the benzene ring could be established. This may be due to the fact that polar substituents decrease the lipophilicity of the benzene ring; thus they reduce the affinity of these substrates and counterbalance any increase in enzymic rates, caused by their electronic effects.

In the present study, we have tested the conversion of several 8-aryl- and 8-pyridylhypoxanthines into the corresponding xanthines by bovine milk xanthine oxidase (Scheme 2). Of special importance are our observations on



Scheme 2. 1: R = C_6H_5; 2: R = *p*-nitrophenyl; 3: R = *p*-anisyl; 4: R = 2′-pyridyl; 5: R = 3′-pyridyl; 6: R = 4′-pyridyl; 7: R = 3′-*N*-methylpyridinium cation; 8: R = 4′-*N*-methylpyridinium cation

8-(*N*-methylpyridinio)purines, bearing a fixed positive charge in the pyridine ring.

Materials and Methods

Hypoxanthines 1—8 were synthesised according to ref. 4; the 1-(14) and 3-methyl derivative 9 of compound 7, and finally the 1-, 3- and 9-methyl homologs of compound 8 according to ref. 5.

The xanthines, resulting from oxidation of compounds 4—8, were prepared according to ref. 4. The xanthines formed by oxidation of compounds 2 and 3 are new and will be described separately. Xanthine 13, obtained by reaction of compound 9 with xanthine oxidase, closely resembles compound 10 in its physical properties (see Table IIA).

Ultraviolet spectra were measured on a Cary 14 recording spectrophotome-

ter; pK values were derived from plots of λ_{max} as function of pH.

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, Molecular Sciences, University of Sussex, England [6]; ratio of activity/E_{450} at 25°C about 120. (2) The commerical milk xanthine oxidase of Sigma Co., Saint Louis, Mo., U.S.A., had 2000 units/ml (55.2 mg of protein/ml) and a ratio of activity/E_{450} at 25°C of about 60. This preparation exhibited essentially the same specificity as the enzyme of Dr. Bray.

Definition of enzyme unit: At pH 8.0 and 29°C, one unit of xanthine oxidase converts 1 μmol/min of xanthine to uric acid, when the concentration of the substrate is $5 \cdot 10^{-5}$ M.

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

Measurement of enzymic rates. All components for the reaction were dissolved in 0.01 M phosphate buffer of pH 8. Substrate and catalase were incubated together for 3 min, and the enzymic reaction was started by adding xanthine oxidase at zero time; total volume was 3 ml. The mixture was placed into the thermospacer of a Cary 14 recording spectrophotometer at 29°C. Controls were run without xanthine oxidase. Readings were taken at the wavelengths, specified in Table I. Rates were derived from the initial linear portion of the curves, showing ΔA as function of time. However, compounds 7—9 made an exception, since they passed first through a latent period before attaining maximal rates (see Fig. 4). Relative rates were calculated by assuming a linear relation between enzyme concentration and rate of oxidation and by assigning always to xanthine the value of 100 at any given concentration of xanthine oxidase. Values of V and K_m were derived from Lineweaver-Burk plots (see Fig. 3). Values of V were also determined directly from a plot of rates versus substrate concentration (see Fig. 1 and Table I).

The pH dependence of enzymic reactions was measured with the following buffers: pH 5—7, 0.05 M citrate/phosphate; pH 8, 0.01 M phosphate; pH 9—10, 0.05 M borate; pH 11, 0.05 M glycine.

The enzymic oxidation products were purified by paper chromatography. The reaction mixture was brought to dryness in vacuo and the residue was extracted with warm dimethylformamide. The extract was spotted on Whatman paper No. 1. The following solvents were used for descending chromatography: A, *n*-butanol/5 M acetic acid (2 : 1, v/v); B, 95% ethanol/12.5% ammonia (4 : 1, v/v); C, isopropanol/dimethylformamide/water (13 : 5 : 2, v/v); D, 95% ethanol/acetic acid/5% HCl (17 : 1 : 1, v/v).

Spots were detected by their fluorescence under a Desaga MinUvis ultraviolet lamp ($\lambda \approx 254$ nm).

For very low relative rates, it was not always possible to determine the standard deviation of the rate because of the large amount of enzyme needed and the larger number of repetitions required (see Table I).

Inhibition experiments. The enzyme underwent half an hour preincubation with the inhibitor, or substrate and inhibitor were added simultaneously at zero time to the enzyme (see Table IV).

TABLE I

ENZYMIC OXIDATION OF 8-SUBSTITUTED HYPOXANTHINES TO XANTHINES

All enzymic reactions at 29° C, pH 8. All rates are expressed as percentage of the rate of xanthine at the same enzyme dilution. Controls were run without the enzyme.

Compound No.	8-Substituent	λ_{max} (nm)		p*K*	Wavelengths used for rate measurement (nm)	Xanthine oxidase (units/ml ($\times 10^{-2}$) concentration)	Relative rate ** (%) (± S.D.)	Nos. of experiments
		N *	A_1 *					
1	Phenyl	286	294	0 9.0 12.5	310	4	1.47 ± 0.11	5
2	*p*-Nitrophenyl	330	362	0.8 7.5 12.0	285 325 363	4	2.0	2
3	*p*-Anisyl	297	305	0.5 9.4 >14	325 330 335	10	0.6	2
4	2'-Pyridyl	300	307	1.8 8.5 12.4	300 340 350	5	1.0	3

5	3′-Pyridyl	290	303	2.8 7.7 13.0	320 330	2	5.3	4
6	4′-Pyridyl	295	311	3.4 7.0 12.4	340	3.3	2.3	4
7	3′-*N*-Methyl-pyridinium cation	305 ***	317 †	5.5 11.7	295 340 355	0.5	64 ± 13	14
8	4′-*N*-Methyl-pyridinium cation	342 ***	366 †	5.5 11.4	390	0.5	51 ± 5	6
9	3-Methyl derivative of compound 7	311—312 ***	325 †	5.0	255 320	5	5.5	2

* N, neutral form; A_1, monoanion.

** Maximal rates from plots of rates versus log (substrate concentration) (see Fig. 1); S.D. = standard deviation.

*** These values refer to the zwitterions, present at pH above 5.5

† These values refer to the zwitterion-anions.

Results

(1) Enzymic oxidation of 8-aryl- and 8-pyridylhypoxanthines

Table I shows that introduction of an electron-attracting (*p*-nitro-, compound 2) or electron-donating substituent (*p*-methoxy-, compound 3) alters the rate of 8-phenylhypoxanthine 1 only little, but compound 3 is attacked about three times slower than compound 2. Presumably the *p*-methoxy group decreases the electrophilicity of C-2, when this position is attacked, e.g. by $-SS^-$ or OH^- [7]. Similarly the rates of the isomeric 8-pyridylhypoxanthines 4—6 are not very different (Table I), but the 2′-pyridyl derivative 4 is oxidised 2—5 times slower than its isomers 5 or 6. Altogether the close range of rates for compounds 1—6 indicates that the main effect of 8-aryl substituents on the enzymic oxidation of hypoxanthines may be ascribed to their size, i.e. these substituents may inhibit the activation process, delineated in Scheme 1.

However, a profound change is observed when the pyridyl nitrogen is quaternised. This conversion is possible only with the 3′- and 4′-derivatives, leading to compounds 7 and 8, but in compound 4 it is prevented by steric interference of a substituent at the ortho nitrogen in the pyridine ring with positions 7 or 9 in the imidazole moiety [5]. Pyridine is a typical π-electron-deficient heterocycle [8] and quaternisation enhances its electron-attracting capacity still further. In addition, the 8-substituent in compounds 7—9 no longer possesses lipophilic character.

Table I shows that quaternisation of compounds 5 and 6 increases the enzymic rates approx. 12- and 22-fold, respectively. Both compounds 7 and 8 exhibit a typical bell-shaped p*S* vs. activity curve (Fig. 1).

At this point we shall consider the dissociation constants (see Table I). For all hypoxanthines studied in the present experiments, it is assumed that monoanion formation involves the imidazole ring, like in hypoxanthine itself [9].

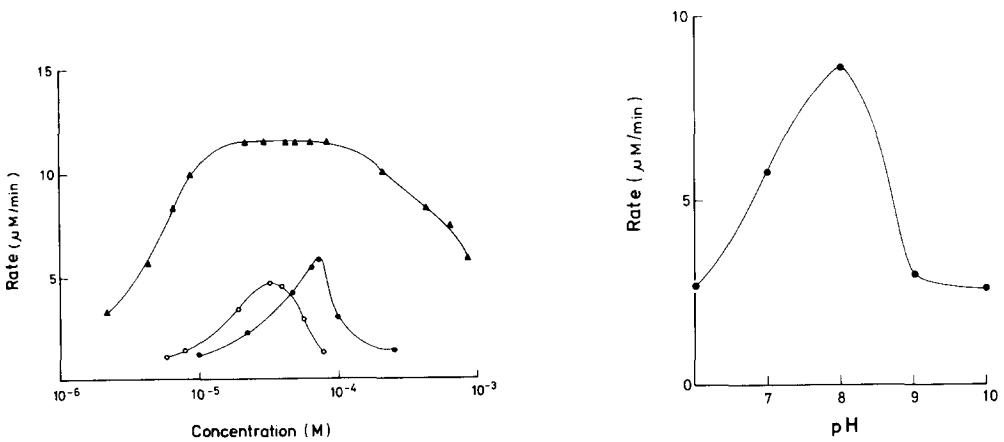


Fig. 1. Rates of oxidation by milk xanthine oxidase as function of substrate concentration. ▲———▲, Hypoxanthine; ●———●, compound 7; ○———○, compound 8. All experiments at pH 8, 29°C; enzyme concentration: $3.3 \cdot 10^{-3}$ units/ml.

Fig. 2. Rate of oxidation of 8-(3′-*N*-methylpyridinium)hypoxanthine betaine 7 as function of pH. Substrate, $5.3 \cdot 10^{-5}$ M; xanthine oxidase, $5 \cdot 10^{-3}$ units/ml; 29°C.

For compound 8 this has been established by comparing the pK values of its N-methyl derivatives (Table IIB). The pK of compound 8 is close to the value of its 1-methyl derivative, the latter undergoing dissociation exclusively in the imidazole ring; ionisation at N-1 in the 9-methyl isomer is characterised by a much higher dissociation constant.

Both compounds 7 and 8 show a large increase in acidity (pK 5.5) relative to compounds 5 and 6, i.e. at their pH optimum of 8 (Fig. 2) they are present to more than 99% as zwitterions (e.g. compound 7a in Scheme 3). However, this fact cannot explain the large increase in rates. For example, at the enzymic pH optimum of 8, compound 6 (pK 7) is present to 90% as anion, but its rate is only 1/30 that of compound 7. Furthermore, we have shown previously that both anions and neutral molecules can serve as substrates for xanthine oxidase [10]. Therefore the high percentage of anions in the solution cannot be the

TABLE II

PHYSICAL PROPERTIES OF 8-SUBSTITUTED XANTHINES AND HYPOXANTHINES

For solvents see Materials and Methods.

(A) 8-Substituted xanthines

Substituents	Form * of purine	λ_{max} (nm)	R_F in solvent	Fluorescence
8-*p*-Nitrophenyl	N	285 380	(C) 0.63	Orange-red
8-(3′-*N*-methylpyridinium (10)	C Z	320 330	(A) 0.12 (D) 0.05	Yellow
8-(4′-*N*-methylpyridinium (11)	C Z	386 362	(A) 0.09 (D) 0.01	Yellow
3-Methyl-8-(3′-*N*--methylpyridinium (13)	Z	239 333	(D) 0.10	Yellow

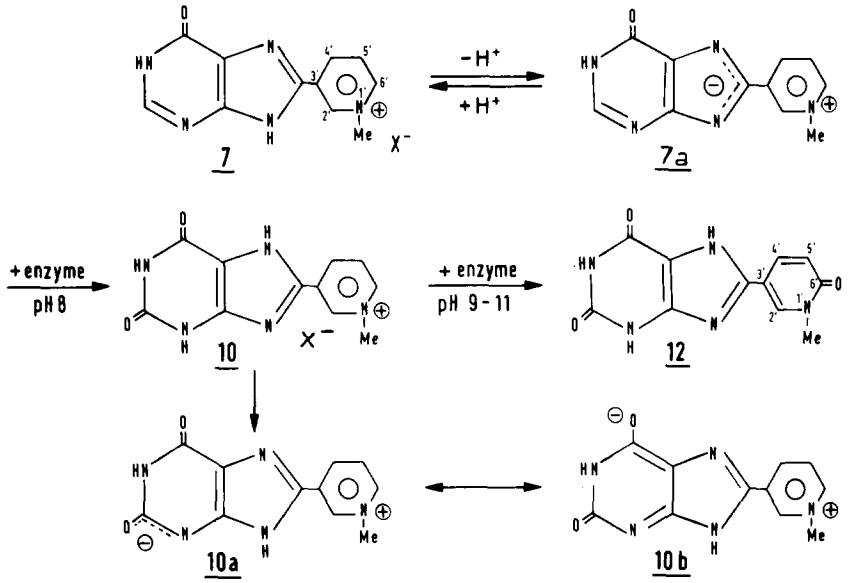
(B) 8-(4′-*N*-Methylpyridinio)hypoxanthines

Substituents	Form * of	λ_{max} (nm)	pK	
			a ***	*b* ***
None (8)	C Z	342 366	—0.5	5.5
1-Methyl	C Z	344 369	—0.3	5.7
3-Methyl	C Z	350 372	+2.0	5.0
9-Methyl	C Z	339 368	—1.2	8.8

* N, neutral form; C, cation; Z, zwitterion.

** For conversion of the dication into the "fixed" monocation.

*** For conversion of the "fixed" monocation into the zwitterion.



Scheme 3.

only factor responsible for the high rates of compounds 7 and 8, although the problem of tautomerisation in the imidazole ring (see Scheme 1) does not arise for anions.

We are thus led to the conclusion that the presence of a fixed positive charge may be responsible for the high rates of compounds 7 and 8, presumably by causing interaction with an anionic site in or near the active enzymic center. Thus the contribution of the pyridinium cation in these two substrates differs fundamentally from the effect of the lipophilic 8-aryl substituents in compounds 1—6.

Compound 9, the 3-methyl derivative of compound 7, yields the xanthine 13, at a rate about 1/12 of that of compound 7 (Table I). It should be recalled that 3-methylhypoxanthine is oxidised only at 1/180 of the rate of hypoxanthine itself [1]. Thus here again the 8-pyridinium substituent has a marked accelerating effect.

The 1-methyl isomer 14 is not converted to the corresponding xanthine derivative, in analogy to the refractoriness of 1-methylhypoxanthine. This is in accord with the reaction mechanism, proposed in Scheme 1.

(2) Inhibition experiments

If substrates 7—9 become attached to an anionic site in or near the active center of xanthine oxidase, then quaternary ammonium salts should be competitive inhibitors. However, neither tetramethylammonium nor *N*-methylpyridinium ion (the latter representing the cationic portion of the quaternary hypoxanthines), at concentrations exceeding 4—5 times those of the substrates, altered the rate of compound 7 or 8 at pH 8 (Table IVA). Furthermore, compound 7 as competitive substrate reduced the rate of xanthine only little (Table IVB), while xanthine inhibited the oxidation of compound 7 markedly. These results agree well with the difference in K_m values for the substrates involved

(Table III). Likewise the quaternary xanthine 11, the oxidation product of compound 8, did not inhibit the conversion of xanthine to uric acid. Similar discrepancies between V and K_m values have been reported recently for other substrates of xanthine oxidase [11].

Table IVA also shows that 8-phenylhypoxanthine 1 and its *p*-methoxy derivative 3 are highly efficient inhibitors of the oxidation of compound 7. Table IVB demonstrates that both compound 1 and 3, as well as 8-phenylxanthine, are powerful inhibitors of the conversion of xanthine to uric acid.

The lack of inhibition by simple quaternary ammonium salts may indicate that the formation of an electrostatic bond between these cationic substrates and an anionic site in the active center of the enzyme is not sufficient to explain their high rates.

(3) Latency of the oxidation of quaternary substrates

A most remarkable aspect of the enzymic reaction of compounds 7, 8 and 9 is the appearance of a lag phase (Fig. 4). The latter is shortened by increasing the temperature and is prolonged by increasing substrate concentration (Table V). The lag phase suggests that these purines induce a relatively slow conformational change in the enzyme molecule. If the oxidation is carried out at 10°C so that the substrate is practically not attacked during the first 20 min, small increases in absorbance are observed at 294 and 390 nm, which are characteristic regions in the spectrum of xanthine oxidase exhibiting decreased absorption, while the enzyme is being reduced [12].

The marked temperature dependence of the lag phase indicates that for the quaternary substrates the conformational change is an integral part of the overall enzymic reaction, i.e. compounds 7, 8 and 9 can be oxidised only after this change has taken place. For reasons set out below, we assume that this involves association of the zwitterionic substrates with an ion pair in or near the active center of the enzyme. Probably as a consequence of the approach of these zwitterions, the electrostatic bond between the two partners of the ion pair is opened, removing a "barrier" and permitting proper attachment of the substrate to form an ES complex. The rate enhancement may then be ascribed to electron attraction by the pyridinium cation, which facilitates nucleophilic attack at C-2.

TABLE III

KINETIC CONSTANTS OF VARIOUS SUBSTRATES OF XANTHINE OXIDASE

All measurements at pH 8.0, 29°C.

Substrate	V *	$[S]_{opt}$ ($\times 10^5$ M)	V **	K_m ($\times 10^6$ M)
Hypoxanthine	117 ± 9	≈5 ***	121	3.3
Xanthine	100	4.3	100	5.25
8-(3′-*N*-Methyl-pyridinio)-hypoxanthine betaine (7)	64 ± 13	7.1	195	125
8-(4′-*N*-Methylpyridinio)-hypoxanthine betaine (8)	51 ± 5	4	250	167

* Relative maximal rates at optimal substrate concentration (see Fig. 1).

** Relative maximal rates, extrapolated from Lineweaver-Burk plots (see Fig. 3).

*** The pS -vs. activity curve of hypoxanthine is characterised by a broad maximum (see Fig. 1).

TABLE IV

INHIBITION EXPERIMENTS

All experiments at pH 8.0, 29°C; enzyme concentration $3.3 \cdot 10^{-3}$ units/ml, as in kinetic runs without inhibitor.

Inhibitor	Preincubation (min)	I_{50} ($\times 10^{-6}$ M)
(A) Substrate 8-(3′-*N*-methylpyridinium)hypoxanthine 7, $4.5 \cdot 10^{-5}$ M		
Tetramethylammonium ion	0	— *
N-Methylpyridinium ion	0	— **
8-Phenylhypoxanthine (1)	0	0.8
8-*p*-Anisylhypoxanthine (3)	30	1.0
Xanthine	0	16
(B) Substrate xanthine, $6.5 \cdot 10^{-5}$ M		
Compound 7	0	100
8-Phenylhypoxanthine (1)	30	0.5
8-*p*-Anisylhypoxanthine (3)	30	0.5
8-Phenylxanthine	30	1.0

* At a concentration of $1.8 \cdot 10^{-4}$ M, no inhibition was observed.

** Ineffective at $2.25 \cdot 10^{-4}$ M.

(4) pH dependence of the enzymic oxidation of compound 7

Oxidation of compound 7 to the xanthine derivative 10 proceeds at its maximal speed at pH 8 (Fig. 2). At pH 6 and 9, the rate is reduced to about one-third, although the substrate is still present to 80 and 99%, respectively, as zwitterion.

When the oxidation of compound 7 was followed under alkaline conditions,

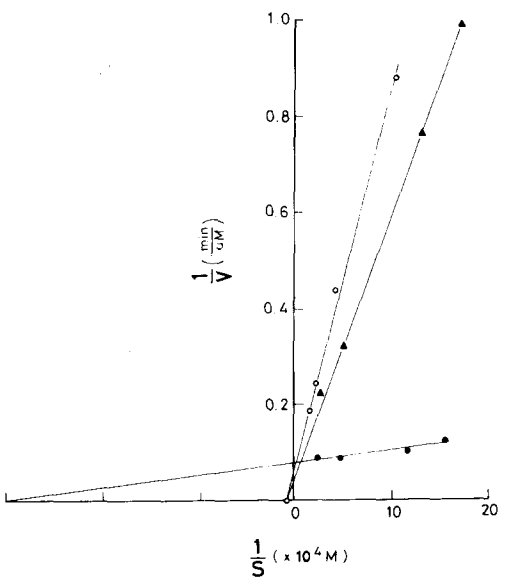


Fig. 3. Lineweaver-Burk plots for hypoxanthine (●———●); compound 7 (▲———▲); and compound 8, (○———○). Xanthine oxidase, $5 \cdot 10^{-3}$ units/ml for all substrates; pH 8.0; 29°C.

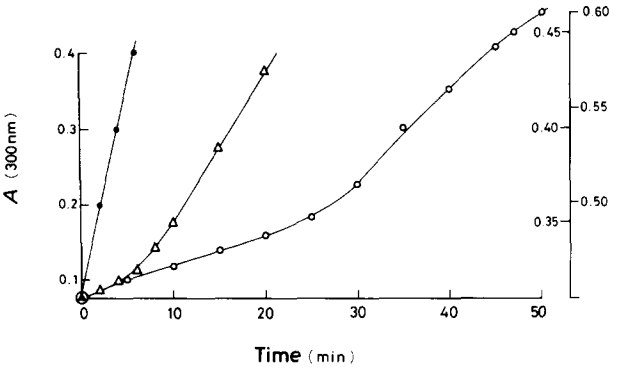


Fig. 4. Lag phase during the enzymic oxidation of 8-(3′-*N*-methylpyridinium)hypoxanthine betaine 7. In all experiments, enzyme concentration was $5 \cdot 10^{-3}$ units/ml; pH 8.0. ●———●, xanthine, $5 \cdot 10^{-5}$ M, 29°C (left-hand ordinate shows absorbance at 300 nm). △———△, compound 7, $4.75 \cdot 10^{-5}$ M, 29°C (inner right-hand ordinate indicates absorbance at 355 nm). ○———○, compound 7, $6.3 \cdot 10^{-5}$ M, 10°C (outer right-hand ordinate for absorbance at 355 nm). Note that xanthine oxidation starts immediately at maximal speed; oxidation of compound 7 increases at 29°C progressively during a period of about 6 min and at 10°C over about 30 min, before the maximal enzymic rate is attained.

e.g. at pH 10, a new phenomenon was observed. The λ_{max} shifted initially from 319 to 330 nm and the absorbance at 315 nm (where the greatest changes were observed) decreased as expected. However, after 2 h, a hypsochromic displacement of λ_{max} started and the absorption at 315 nm increased again (Fig. 5). After approx. 30 h, the reaction ceased to progress any further. The maximum was now at 311 nm, and the absorbance at 315 nm had increased by about 70%

TABLE V

LAG PHASE IN THE ENZYMATIC OXIDATION OF 8-(*N*-METHYLPYRIDINIO)HYPOXANTHINE BETAINE 7.

(A) Influence of temperature on lage phase *

T (°C)	Lag phase (min)	Maximal rate of oxidation (μM/min)
4	— **	0.04
10	20—30	0.39
29	2— 3	2.26

(B) Concentration dependence of lag phase at 29°C

Concentration of compound 7 (X10^5 M)	Lag phase (min)	Maximal rate of oxidation (μM/min)
1.05	0	1.30
5.25	4	2.26
10.5 ***	13	1.26
52.5 ***	30	0.17

* Substrate $5.25 \cdot 10^{-5}$ M, enzyme (Sigma) $5 \cdot 10^{-3}$ units/ml; pH 8.0.

** At 4°C, the rate of oxidation is too slow to permit a clear distinction between lag phase and linear progress of the reaction.

*** These concentrations represent points on the descending branch of the curve, showing rates as function of log (substrate concentration) (see Fig. 1).

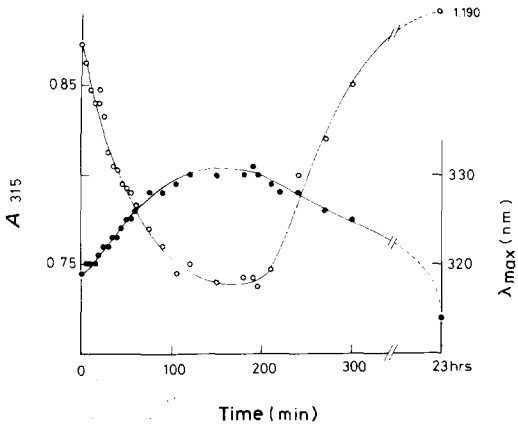


Fig. 5. Two-step oxidation of compound 7 at pH 10. Xanthine oxidase, $5 \cdot 10^{-3}$ units/ml; substrate, $5.25 \cdot 10^{-5}$ M; temperature, 29°C. ●———●, change of λ_{max} (right-hand scale); ○———○, change of absorbance at 315 nm (left-hand ordinate).

(Fig. 5). Paper chromatography revealed the presence of two oxidation products (Table VI): The quaternary xanthine 10 and a new compound 12. The latter derivative was also formed when compound 10 was exposed directly to enzymic oxidation in the pH range 9—11; however at pH 8, compound 10 was refractory to further enzymic attack. It is evident that compound 12 must be a xanthine derivative and that the second oxidative step, discernible in Fig. 5, must involve the pyridine ring (see Scheme 3).

Final proof of structure 12 has to await synthesis of this purine, but the formula assigned in Scheme 3 is supported by the following data (Table VI): (a) The λ_{max} of compound 12 is at a shorter wavelength than that of compound 10, suggesting that the cationic group has been lost. (b) Even in strongly

TABLE VI

COMPARISON OF PURINES, BEARING AN 8-(3′-*N*-METHYLPYRIDINIO) OR AN 8-(3′-*N*-METHYL-6′-OXOPYRIDYL) SUBSTITUENT

Compound No.	Compound	Form * of purine	λ_{max} (nm)	R_F in solvent D	Fluorescence
10	8-(3′-*N*-Methylpyridinio)-xanthine	Z	330 249 sh	0.05	Yellow
12	8-(3′-*N*-Methyl-6′-oxopyridyl)-xanthine	A_1	312	0.19	Violet
14	1-Methyl-8-(3′-*N*-methylpyridinio)-hypoxanthine	Z	320 236	0.10	Blue
15	1-Methyl-8-(3′-*N*-methyl-6′-oxopyridyl)-hypoxanthine	(pH 7) **	280	0.34	Violet

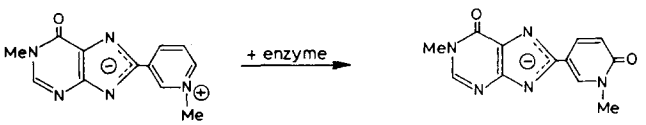
* Z, zwitterion; A_1, monoanion.

** After extraction of pure compound 15 from the paper chromatogram, the amount of material was insufficient for determination of p*K* values.

acid media (solvent D), compound 12 exhibits a large R_F, as indication that it does not carry a fixed positive charge. (c) The quaternary xanthines 10, 11 and 13 show intense yellow fluorescence, while that of compound 12 is brilliant violet.

We have also tested the behaviour of compounds 11 and 13 at alkaline pH, but these xanthines were not attacked further by the enzyme. Likewise the *N*-methylpyridinium cation itself proved refractory.

On the other hand, 1-methyl-8-(3′-*N*-methylpyridinio)hypoxanthine betaine 14, which is not converted into the corresponding xanthine (see above), is oxidised to a new purine, assumed to be the corresponding α-pyridone 15 (Scheme 4). While enzymic attack at position 2 of an 8-substituted hypoxanthine is al-



14 15

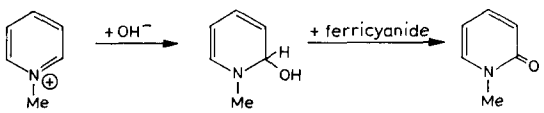
Scheme 4.

ways accompanied by a bathochromic shift of λ_{max}, conversion of compound 14 to 15 causes a hypsochromic displacement of about 40 nm (Table VI). Again final proof of the structure of compound 15 has to await independent synthesis.

In view of the unusual pH dependence of the reaction of compound 10 → compound 12, it is noteworthy that oxidation of compound 14 at pH 8 (relative rate ≈0.3) is approximately three times faster than at pH 10.

(5) Do the quaternary xanthines 10, 11 and 13 form pseudo-bases?

In alkaline media, many heteroaromatic cations add hydroxyl ion to give dihydro derivatives, the so-called "pseudo-bases" (Scheme 5) [13]. This process



Scheme 5.

finds its expression in a hypsochromic shift of λ_{max} and in the appearance of signals of non-aromatic protons (δ = 5—6 ppm) in the NMR spectrum [14]. The pseudo-bases are easily oxidised by ferricyanide to the corresponding cyclic amides (Scheme 5) [15].

In contrast, the absorption maximum of compounds 10 and 11 shows a steady displacement to longer wavelengths in the range of pH 8—14 [4]. Likewise the NMR spectrum of these two purines at pH 14 reveals only the presence of the aromatic protons of the pyridinium moiety, δ = 8.3—9.5 ppm. Thus we conclude that in aqueous solutions of the quaternary derivatives tested here, no spontaneous pseudo-base formation takes place, but these observations do not exclude the possibility that such a change may be promoted by the enzyme.

Discussion

The present study reveals several special features of the enzymic oxidation of purines, bearing a quaternary pyridyl substituent at position 8:

(1) pH dependence of enzymic oxidations

Conversion of the hypoxanthines 7 and 8 into the corresponding xanthines exhibits an optimum at pH 8. In the pH range 8—9, the structure of the zwitterions of compounds 7 and 8 (pK 5.5) remains practically unchanged. Therefore the curve of Fig. 2 permits a more detailed interpretation. We have reported previously that 1,6,7-trimethylpteridin-4(3H)-one, a substrate that cannot form an anion, shows a sharp decrease of its rate from pH 8 to 9 [10]. For this part of the pH vs. activity curve, a pK of about 8.6 was derived. A similar value (p$K \approx 8.8$) can be obtained from the right-hand portion of Fig. 2. It is thus concluded that the decline of rates above pH 8, observed for compounds 7 and 8, is essentially due to pH-dependent changes in the enzymic center.

During 50 h observation, no attack of xanthine 10 by the enzyme could be detected at pH 8, but the reaction became measurable by raising the pH to 9—10.5. A similar behavior has been reported for quaternary substrates, derived from pyridine or quinoline [16,17]. It was concluded that substrates like *N*-methylnicotinamide cation attach themselves to an anionic site in or near the enzymic center of xanthine oxidase, characterised by an apparent pK of about 10.7, and that "the effect of high pH on the substrate specificity of xanthine oxidase appears to be due solely to a change at the substrate binding site" [16]. Murashige et al. [17] proposed that a protonated group in the active center must lose its charge before cationic substrates can be bound to a nearby anionic site in the enzyme. This means that in the enzymic center an ion pair is present. After the positively charged partner of the ion pair has been eliminated, it becomes possible for a cationic substrate to approach in such a way that its heterocyclic moiety, bearing the charge, can be attacked. Presumably the group representing the positive charge in the enzyme is an ammonium ion. Indeed it has been reported that certain inhibitors like 2,4-dinitrofluorobenzene or benzaldehyde react with a specific amino group of xanthine oxidase only at elevated pH ($\approx$10.8), i.e. when the amino group has been deprotonated [18].

While the quaternary xanthine 10 behaves like the cationic substrates of Greenlee and Handler [16], conversion of the hypoxanthine 14 to the 2-pyridone 15 is actually faster at pH 8 than at 10 or 11. This discrepancy may be explained by differences in ionisation. We assume that the xanthine 10 follows the same dissociation sequence as xanthine itself, i.e. N-3 → N-9(7) [19]. The zwitterion of compound 10 can be represented as compound 10 a,b, in which the negative charge is mainly located in the pyrimidine ring. The anion-zwitterion of compound 10, in which also the imidazole moiety bears a negative charge, shows pK 10.4 [5]. In the hypoxanthine 14, however, the negative charge of the zwitterion (pK 5.7) is concentrated mainly in the imidazole ring. It may be proposed tentatively that the smaller distance between the opposite charges in compound 14 permits smooth interaction with the ion pair in the enzymic center already at pH 8. On the other hand, attack at the pyridinium moiety of compound 10 requires both dissociation of the NH group in

the imidazole ring and progressive deprotonation of the ammonium ion in the active center with increasing pH.

(2) The lag phase in the oxidation of the quaternary hypoxanthines 7, 8 and 9

Oxidation of the hypoxanthines 7, 8 and 9 reaches a constant rate only after a lag phase. Such delays can be observed only for reactions which are sufficiently rapid. In all three cases, the enzymic oxidation is inhibited by excess substrate, i.e. by formation of an ES_2 complex. We have no information whether such a complex prevents the required conformational change or inhibits the oxidative process itself. The interesting problem arises whether in the ES_2 complex each quaternary substrate molecule interacts with a separate anionic site.

(3) Mechanism of oxidation of the N-methylpyridinium moiety

According to Edmondson et al. [7], the first step in the oxidative reactions of xanthine oxidase consists in the nucleophilic attack of an RSS^- group in the active center on a ring carbon in the substrate. If the same mechanism applies to all heterocyclic substrates, then the *N*-methylpyridinium moiety in compound 10 and 14 is attacked directly at a carbon atom, neighboring the positively charged nitrogen. Such a process has been proposed previously in order to explain the enzymic oxidation of 8-methyllumazine at position 7 [10].

The refractoriness of the xanthine 11 to attack at its pyridinium moiety is noteworthy. Greenlee and Handler [16] observed that, with one possible exception, only heterocyclic cations with a free paraposition, relative to the ring nitrogen, and with a substituent in metaposition, were oxidised to 2-pyridones or 2-quinolones. Our results with compounds 10 and 14 and with 8-methyllumazine are in accord with these observations. However, the resistance of xanthine 13 to further oxidation indicates that also position 3 in the purine ring must be available for formation of the proper ES complex.

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