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OXIDATION OF *N*-METHYL SUBSTITUTED HYPOXANTHINES, XANTHINES, PURINE-6,8-DIONES AND THE CORRESPONDING 6-THIOXO DERIVATIVES BY BOVINE MILK XANTHINE OXIDASE

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Summary

1. The oxidation of six series of purines (hypoxanthines, xanthines, purine-6,8-diones and the corresponding 6-thioxo derivatives) by a highly purified bovine milk xanthine oxidase (EC 1.2.3.2) has been studied, using a variety of *N*-methyl derivatives.

2. *N*-Methyl substituents can either enhance or reduce enzymic rates. Enhancement is ascribed to blockade of groups which mediate unfavorable modes of binding of substrate to enzyme. Introduction of *N*-methyl groups can also inhibit enzymic oxidation, either by occluding essential binding groups or by preventing spontaneous or enzyme-induced tautomerisation processes, which create suitable binding sites in the substrates.

3. In all purines which are rapidly attacked by xanthine oxidase, proper attachment to the active center is mediated by the groupings (3)NH, (9)N or (3)N, (9)NH.

4. Reduced rates usually express lowered substrate affinity, which finds its expression in weak competitive inhibition of xanthine oxidation.

Introduction

Enzymic oxidation of *N*-methylpurines has been used previously to obtain information about the "active" structures of purines in the enzyme-substrate complex with xanthine oxidase (EC. 1.2.3.2) [1]. It was assumed that the enzymic center binds purines by specific groups and that activation processes may involve (besides change of bond lengths and bond angles) also "induced" tautomerisations. In the present study, we report some new observations on 6-

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oxopurines and extend these experiments to 6-thioxopurines and their *N*-methyl derivatives.

Materials and Methods

Hypoxanthines. All members of this series were prepared as described in refs. 2—8.

6-Thioxopurines. Syntheses according to ref. 4. [8-^{14}C]6-Thioxopurine was purchased from New England Nuclear, Boston, Massachusetts.

4,5-Cyclopentenopyrimidines. (i.e. 4,5-trimethylenepyrimidines) [9] were a gift of Dr. B.R. Baker, Stanford Research Institute, Menlo Park, California.

Xanthines. For synthetic methods see ref. 10.

6-Thioxanthines. See ref. 11.

Purine-6,8-diones. See ref. 12.

6-Thioxopurin-8-ones. See ref. 13.

Uric acids. For synthesis of 1-methyl-, 7-methyl- and 1,7-dimethyluric acids see refs. 14 and 15.

6-Thiouric acids. 1-Methyl-6-thiouric acid [16] and its 3-methyl isomer [17] have been described previously. The 7-methyl and the 1,7-dimethyl derivative were obtained by enzymic oxidation of compounds 20 and 21 and were identified by their spectral properties.

The purity of all substrates used was checked by paper chromatography in three solvents and by quantitative ultraviolet spectrophotometry.

Xanthine oxidase. Highly purified xanthine oxidase from cow milk was a gift of Professor R.C. Bray, Dept. of Biochemistry, University of Sussex, England [18]. The enzyme was stabilised by addition of 1 mM sodium salicylate and showed a ratio activity/$E_{450} \approx 120$. When diluted 1 : 30 000 and incubated with $6.6 \cdot 10^{-5}$ M xanthine at pH 8.0 and 29°C, the enzyme produced 1.2 µg/ml/min of uric acid. At this dilution, the reaction mixture contains approximately 3.8 µg/ml protein. Assuming molecular weights of 310 000 or 360 000 [19,20], the enzyme concentration is $1.1—1.2 \cdot 10^{-8}$ M.

The enzyme was stored at −20°C. Prior to use, the stock solution was diluted 1 : 100 with 0.01 M phosphate buffer, pH 8.0. When this dilution was kept at +4°C, in the presence of chloroform, its activity decreased slowly with a half time of about two weeks.

The new enzyme preparation showed essentially the same substrate specificity, as was reported with an earlier sample of xanthine oxidase [1], but the following differences were observed: 7-Methylxanthine (compound 15) has previously been found to be refractory, but the new enzyme converted it to 7-methyluric acid at a rate about 1/10 of the oxidation of xanthine (see Table III). 3-Methylpurine-6,8-dione (compound 23) reacted very slowly with the older enzyme [21], but resisted attack by the new one. Similarly, 1-methyl-6-thioxopurin-8-one was not oxidised by the new enzyme, in contrast to an earlier report [6]. A quantitative difference was observed with 1-methylxanthine (compound 13): Previously, its rate was found about ½ that of xanthine [1], but the new enzyme oxidised compound 13 approx. 1.4 times faster than xanthine (see Table III).

The reason for these discrepancies are not clear. The substrates used in our

earlier studies were all chromatographically pure. It may be that removal of certain contaminants (e.g. inactive flavin-protein complexes) changes substrate specificity of xanthine oxidase to a certain extent.

Determination of enzymic rates

The reactions were followed by placing the solutions into the thermospacer of an ultraviolet spectrophotometer, after addition of catalase (Worthington), final dilution 1 : 500. Readings were taken at the wavelengths specified in Tables II—IV. The enzymic rates were derived from the initial linear portion of the curves, showing ΔA as function of time. The value 100 was assigned to the rate of xanthine. Relative rates were calculated by assuming a linear relation between enzyme concentration and rate of oxidation for all substrates. For instance if a purine is oxidised by an enzyme dilution of 1 : 1000 and its rate is given as 1%, this indicates that xanthine at the same dilution of xanthine oxidase would be oxidised 100 times faster, although actually its rate was measured at a dilution of 1 : 30 000 (Table III). V and K_m values were obtained from Lineweaver-Burke plots (Fig. 1).

Enzymic oxidation products were purified by paper chromatography. The reaction mixture was brought to dryness in vacuo and the residue extracted with warm Me_2SO. The extract was spotted on Whatman paper No. 1. The fol-

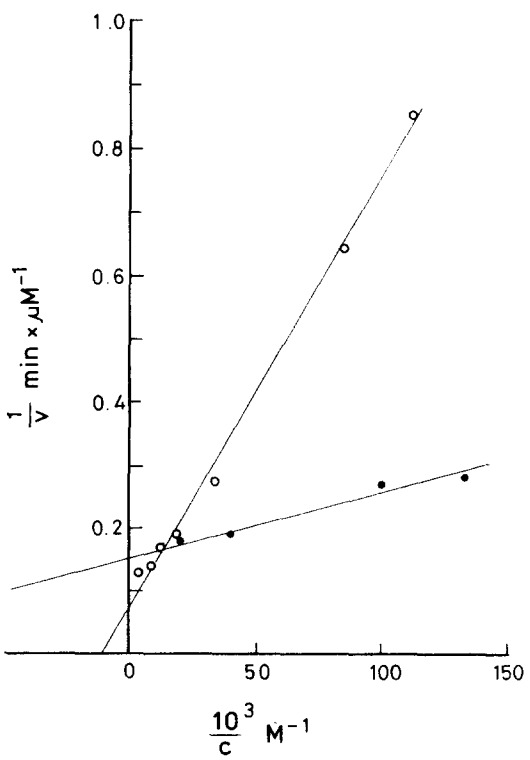


Fig. 1. Lineweaver-Burke plots for enzymic oxidation of (●———●) purine-6,8-dione, compound 22, and (○———○) 7-methyl-6-thioxopurin-8-one, compound 27. Xanthine oxidase 1 : 5000; 0.01 M phosphate buffer, pH 8.0.

lowing solvents were used for descending chromatography: A_1, *n*-butanol/5 M acetic acid (67 : 33, v/v); A_2, 95% ethanol/glacial acetic acid/water (17 : 1 : 2, v/v); B, 95% ethanol/ 25% ammonia/water (8 : 1 : 1, v/v); C, isopropanol/ dimethylformamide/water (13 : 5 : 2, v/v).

In dioxopurines or thioxopurinones, only one position is available for attack, i.e. the reaction product is known in advance. For hypoxanthines and 6-thioxopurines, however, two pathways are open for the first reaction step. In this case, both possible intermediates and the common end-product of the second oxidation step were used as markers. Spots were localised with the aid of a Desaga MinUvis lamp $\lambda \approx 254$ or 366 nm).

Enzymic rates were measured down to 0.01%, i.e. 1/10 000 of the rate of xanthine oxidation. Substrates which were attacked at still lower rates, are usually recorded as refractory, unless after 4—5 days of incubation with the enzyme the oxidation products could be detected on paper chromatograms.

High-voltage electrophoresis

The two possible intermediates in the conversion of [8-^{14}C]6-thioxopurine to 6-thiouric acid, viz. 6-thioxopurin-8-one (compound 11) and 6-thioxanthine, compound 17 (see scheme 2) could not be separated efficiently by paper chromatography. For unequivocal identification of the intermediate 11, high voltage electrophoresis (7.5 V/cm/h) on 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; Sørenson's phosphate buffer, pH 6.6, was used as medium. After completion of the electrophoresis, the paper strip was dried and then left in contact with Ilfex R-100 X-ray film for 2 weeks. The following mobilities (cm/h) were found (average of 5 measurements): 6-thioxopurine approx. 0; compound 11, 10.7 ± 1.6; 6-thioxanthine, 15.5 ± 0.6; 6-thiouric acid, 17.4 ± 1.2. Since the spots were very compact, satisfactory separation of all possible components of the reaction mixture, especially of compounds 11 and 17, was achieved.

Inhibition experiments

Substrate, inhibitor and catalase were incubated with buffer for 3 min, and xanthine oxidase was added at zero time. The rate was compared with a control, containing only substrate and enzymes.

Results

(1) Values of pS_{opt}, V and K_m (Table I)

For all substrates reacting at relative rates above 1%, pS-activity curves were measured. All showed pS optima near $5 \cdot 10^{-5}$ M. The only exceptions were 7-methylpurine-6,8-dione (compound 24) and the corresponding 6-thioxo derivative (compound 27), for which the pS optimum was found at $3 \cdot 10^{-4}$ M. Fig. 2 shows two types of pS-activity curves, with sharp or broad maximum. The shape of the curves is related to auto-inhibition. For instance, 7-methyl-6-thioxanthine (compound 20) is oxidised at half its maximal rate at a concentration of $1.25 \cdot 10^{-4}$ M, which is about twice the $[S]_{opt}$. Purine-6,8-dione (com-

TABLE I

KINETIC CONSTANTS FOR OXIDATION OF PURINES, CATALYSED BY MAMMALIAN XANTHINE OXIDASE.

No.	Compound	$[S]_{opt}$ * ($\times 10^5$ M)	V **	K_M ($\times 10^6$ M)
1	Hypoxanthine	2–8 (b)	108	3.3
5	8-Methylhypoxanthine	4 (b)	94	11.1
12	Xanthine	4.3	100	5.25
13	1-Methylxanthine	5–10 (b)	141	143
15	7-Methylxanthine	(>100) ***	(86)	770
17	6-Thioxanthine	4.5 (b)	57	4.6
18	1-Methyl-6-thioxanthine	7.0 (b)	155	56
20	7-Methyl-6-thioxanthine	6.5	129	32
22	Purine-6,8-dione	5–10 (b)	76	7.3
24	7-Methylpurine-6,8-dione	25 (b)	287	28.8
11	6-Thioxopurin-8-one	6.5	94	25.7
27	7-Methyl-6-thioxopurin-8-one	20	105	83.8

* (b) indicates a broad maximum in the pS-activity curve (see Fig. 2)

** Percent of V of xanthine

*** The values of $[S]_{opt}$ and of V are in doubt because 10^{-3} M represents the limit of solubility of compound 15.

pound 22) also shows $[S]_{opt} \approx 6 \cdot 10^{-5}$ M, but even at a 10-fold higher concentration the rate is still 82% of V. Similar discrepancies are manifest on the left-side branches of the curves in Fig. 2. These differences are reflected in the data of Table I. While the values of V differ about five-fold, the K_m values cover the wide range of $3 \cdot 10^{-6}$ M to $1.4 \cdot 10^{-4}$ M.

The pH optimum was found to be 8.0 for xanthine (compound 12), 6-thioxanthine (compound 17) and their 7-methyl derivatives (compound 15) and (compound 20). The same result has been reported previously for purine, xanthine and purine-6,8-dione [22]. Since the percentage of anions at pH 8 varies for these substrates between 12 and 98%, it is concluded that the enzyme

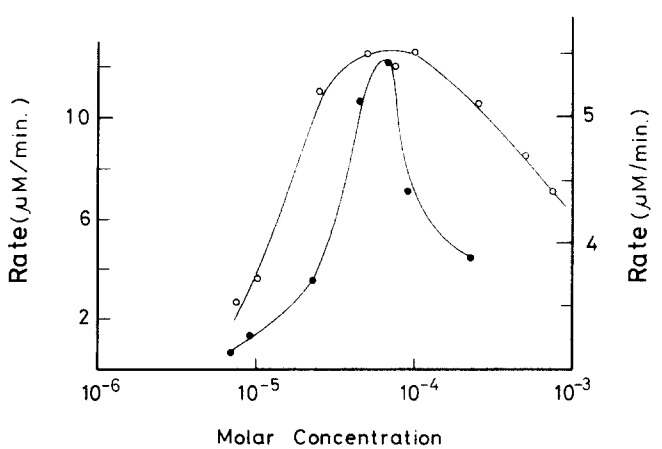


Fig. 2. pS-activity curves of (○———○) purine-6,8-dione (compound 22) (right ordinate) and (●———●) 7-methyl-6-thioxanthine (compound 20) (left ordinate). Xanthine oxidase 1 : 5000; 0.01 M phosphate buffer, pH 8.0.

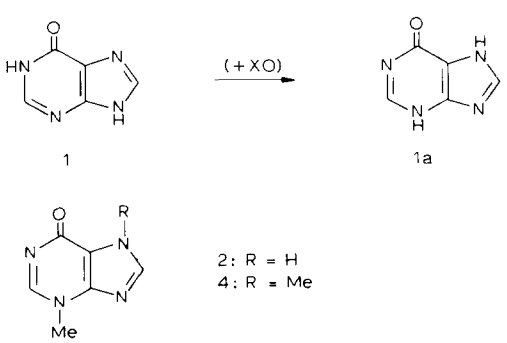
can attack its substrates either as neutral molecules or as anions. This conclusion is strongly supported by the fact that xanthine oxidase attacks substrates which are completely ionised at pH 8, as well as compounds which cannot form anions at all [23]. In either case, the pH optimum of the enzymic reaction is at 8.0.

(2) Hypoxanthines and 6-thioxopurines

At pH 8, both hypoxanthine (compound 1) and 6-thioxopurine (compound 7) are present as mixtures of uncharged molecules and anions (see Table II). Therefore we shall discuss both these forms as possible substrates.

As reported earlier [1], among all mono-*N*-methyl derivatives of hypoxanthine, only the 3-methyl isomer (compound 2) is attacked by xanthine oxidase [1], while all *N,N'*-dimethyl derivatives, including the 3,7-dimethyl isomer compound 4, are refractory (Table II). From the parallel behavior of compounds 1 and 2, it was concluded that hypoxanthine tautomerises in the enzyme-substrate complex to the 3-*NH*-form. Considering first the neutral molecule of compound 1, this tautomerisation involves a simultaneous shift of the NH- group in the imidazole ring from position 9 to 7 (compound 1a in scheme 1) [7]. The refractoriness of the 7-methyl derivative 3 indicates that also binding via the grouping (6)C = 0, (7)NH is important. Thus we may assume that the uncharged molecule of hypoxanthine attaches itself both via (6)C = 0, (7)NH and via (3)NH, (9)N, while compound 2 can utilise only the former site for binding. This would explain the much lower affinity of compound 2 for the active center of xanthine oxidase, as demonstrated by its low enzymic rate and by the observation that oxidation of compound 1 is not slowed down in the presence of two molar equivalents of compound 2. Likewise the 1-methyl derivative is refractory, since it can not tautomerise and thus lacks the characteristic binding sites of compound 1a. It is also understandable that the 3,7-dimethyl derivative 4, in which both binding sites are blocked by *N*-methylation, does not serve as substrate of xanthine oxidase, although its structure resembles compound 1a. Like compound 2, it is also not an inhibitor.

Scheme 1.



On the other hand, the 8-position does not appear to play any role in the formation of the enzyme-substrate complex of hypoxanthine. This follows from the similar rates of oxidation for the pairs hypoxanthine/8-methylhypo-

TABLE II

ENZYMIC OXIDATION OF HYPOXANTHINES, 6-THIOXOPURINES AND RELATED HETEROCYCLIC COMPOUNDS

No.	Compound	λ_{max} (nm)		pK for anion formation	Anion at pH 8 (%)	Wave length used for measurement (nm)	Dilution of xanthine oxidase	Position attacked	Relative rate	Number of experiments
		N *	A *							
	A. Hypoxanthines **									
1	—	249	258	8.4	28.6	280	1 : 30 000	2	108	4
2	3-Methyl-	264	264	8.4	28.6	245	1 : 6 000	2	0.6	2
3	7-Methyl-	256	262	9.4	3.9	w.s. ***	1 : 250	—	—	2
4	3,7-Dimethyl-	264	—	—	—	w.s.	1 : 250	—	—	2
5	8-Methyl-	252	252	7.3	83.3	290	1 : 20 000	2	94	2
6	3,8-Dimethyl	265	268	—	—	270 245	1 : 5 000	2	0.6	2
	B. Thioxopurines **									
7	—	322	312	7.5	76	345	1 : 2 500	8	1	6
8	3-Methyl-	338	332	7.8	61.3	w.s.	1 : 500	—	—	2
	C. 4,5-Cyclopentenopyrimidines									
9	4-Oxo-	264 233	262 234	8.7	16.7	270	1 : 1 000	2	0.9	2
10	4-Thioxo-	325 284	298 222	7.3	83.3	340	1 : 1 500	2	0.05	2

* N, neutral form; A, monoanion.

** Other substrates which were found refractory after incubation with xanthine oxidase, dilution 1 : 250, for 4—5 days, are: 1- and 9-methylhypoxanthine; all *N*,*N*′-dimethylhypoxanthines; all *N*-methyl-6-thioxopurines.

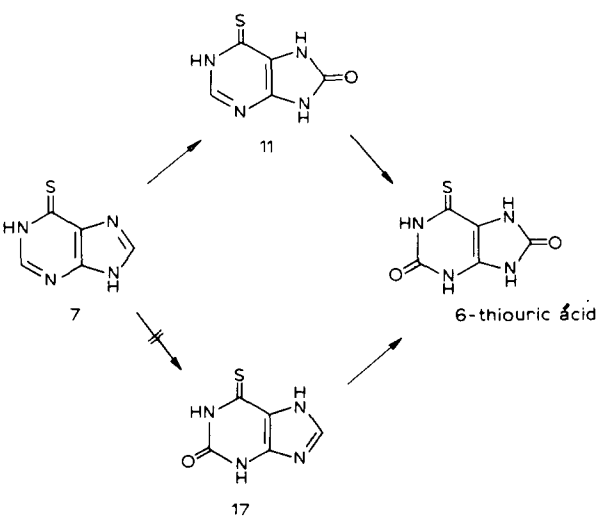
*** w.s., whole spectrum.

xanthine (compound 5) and 3-methyl-/3,8-dimethylhypoxanthine (compound 6) (Table II).

It has been shown that the pathways of the enzymic reaction of compound 1 and of 6-thioxopurine (compound 7) are different [24]. This was concluded from the spectral changes during the oxidation of compound 7 to 6-thiouric acid, but the intermediate 6-thioxopurin-8-one (compound 11, scheme 2) was not identified directly, because of its low stationary concentration. We have now repeated this reaction with [8-^{14}C]6-thioxopurine and have separated the components of the mixture by high-voltage electrophoresis at pH 6.6 (see Materials and Methods). No trace of 6-thioxanthine (compound 17) was found, compound 11 being the only intermediate at any stage. These observations confirm our earlier conclusions about the oxidative pathway of compound 7. The relative rate for the conversion compound 7 → compound 11 was about 1, similar to the results of previous measurements [24,25].

If the uncharged molecules of compounds 1 and 7 are compared first, then their different pathways may be explained by the fact that tautomerism of 6-thioxopurine in the enzyme-substrate complex, analogous to conversion of compound 1 → 1a in scheme 1, is lacking. Therefore compound 7 attaches to xanthine oxidase via the (3)N, (9)NH- grouping, i.e. it forms the "reversed" combination with the active enzyme center. This is illustrated in scheme 3 by assuming that an amide group of the enzyme is responsible for binding to this portion of the substrate molecule.

Scheme 2.



Scheme 3.

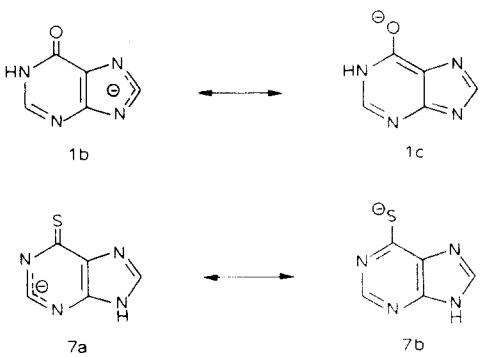
Mode of binding via (3)NH, (9)N

Mode of binding via (3)N,(9)NH

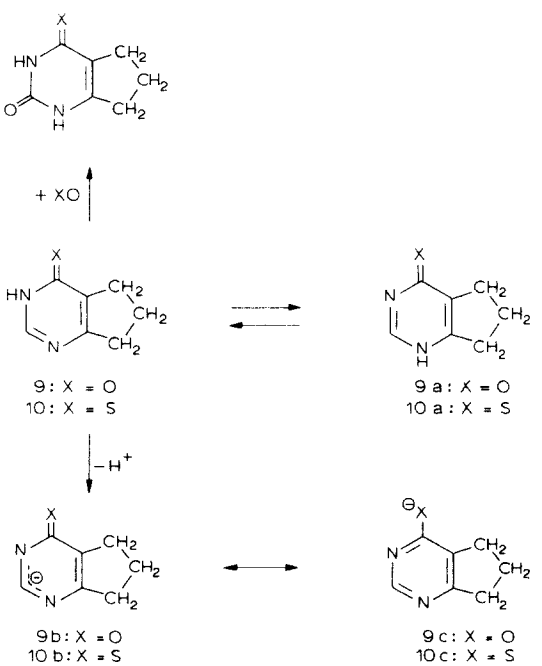
On the other hand, if the anions are the preferred forms of the substrates 1 and 7, we should remember that monoanion formation in compound 7 involves the 1-NH group, in contrast to dissociation of the imidazole-NH in hypoxanthine (scheme 4) [2,7]. If the enzymic process consists in abstraction of a hydride ion from the purine and concerted nucleophilic attack by OH^- [26], the latter may be directed towards the carbon atom that is most remote from the negative charge in the anionic substrate. This would explain the opposite pathways of oxidation of compounds 1 and 7. In the anion of compound 1, again enzymic tautomerisation to the 3-NH form is assumed.

The above conclusions find support in experiments with the 4,5-cyclopentenopyrimidines 9 and 10 (scheme 5). In these heterocyclic compounds,

Scheme 4.



Scheme 5.



only position 2 is available for oxidation. The hypoxanthine analog 9 is oxidised at about 1/100 the rate of compound 1 (see Table II). The pK of compound 9 is 8.7, i.e. at pH 8 only about 17% of anions 9b, c are present. If the neutral molecule serves as substrate, the driving force for tautomerisation to compound 9a is missing since the partial structures, responsible for binding of hypoxanthine to the enzyme, are not available. If the anion 9b, c is attacked by xanthine oxidase, the localisation of the charge in the pyrimidine moiety would obstruct the approach of OH^- and thus would reduce the rate.

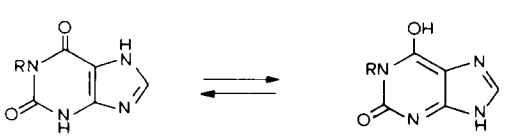
Similar considerations apply to the 6-thio derivative 10, which, in contrast to compound 7, is attacked at C-2. Again the neutral form of compound 10 would be unable to undergo "induced" tautomerisation to compound 10a. However at pH 8, compound 10 is ionised to about 83% (Table II). Therefore the anion 10b,c is the more probable substrate. Again the negative charge in the pyrimidine ring of compound 10 would reduce the rate of attack of OH^- at C-2 sharply.

(3) Xanthines and 6-thioxanthines

The new enzyme preparation oxidises 1-methylxanthine (compound 13) and 7-methylxanthine (compound 15) (Table III). It is remarkable that compound 13 is attacked faster than xanthine (compound 12) itself. For an interpretation of this observation we should recall that the xanthine molecule offers a much larger variety of possible tautomers than hypoxanthine. Let us first consider the neutral forms of compounds 12 and 13 and let us assume that replacement of 1-NH by 1-NMe prevents "unfavorable" attachment of xanthine oxidase via (1)NH, (6)C = 0 or (1)NH, (2)C = 0. Then in compound 13 proper binding of the substrate via the groupings (6)C = 0, (7)NH and (3)NH, (9)N would be enhanced. Alternatively, if rapid attack at position 8 requires the configuration (3)N, (9)NH, as was assumed for compound 7, the neutral molecule xanthine may react in the tautomeric form 12a (scheme 6), in which also the additional grouping (6)C-OH, (7)N may become attached to the active center. In this case again a 1-methyl substituent would prevent "improper" binding via the grouping (1)NH, (2)C = 0.

Regarding the anions of compounds 12 and 13 as preferred substrates of xanthine oxidase, Pfleiderer and Nübel [27] have shown that dissociation of the 3-NH-group in xanthine is accompanied by a proton shift from position 7 to 9 (scheme 7, compounds 12b and 13b). Therefore the anions can bind to the enzymic center via the grouping (3)N, (9)NH and probably also via (6)C-O^-, (7)N, as in the resonance forms 12c and 13c (see scheme 7). Again a 1-methyl substituent prevents improper binding. In compounds 12b,c and 13b,c the

Scheme 6



12: R = H
13: R = Me

12a: R = H
13a: R = Me

TABLE III

ENZYMIC OXIDATION OF XANTHINES AND 6-THIOXANTHINES

No.	Compound	λ_{max} * (nm)		p*K* for anion formation	Anion at pH 8 (%)	Wave length used for measurement (nm)	Dilution of xanthine oxidase	Relative rate	Number of experiments
		N	A						
	A. Xanthines **								
12	—	267	276	7.5	76	300	1 : 30 000	100	20
13	1-Methyl-	268	277	7.9	55.7	300	1 : 30 000	141	8
14	3-Methyl-	272	275	8.5	24	290	1 : 3 000	—	3
15	7-Methyl-	268	287	8.4	28.6	300	1 : 3 000	86	9
16	1,7-Dimethyl-	268	289	8.6	21.1	300	1 : 3 000	0.03	2
	B. 6-Thioxanthines **								
17	—	342 265	342 250	6.2	98.4	325	1 : 15 000	57	7
18	1-Methyl-	338 271	340 248	6.7	95.2	270	1 : 30 000	155	4
19	3-Methyl-	345 337 (sh) 265	338 252	7.9	55.7	360	1 : 1 000	0.01	3
20	7-Methyl-	343 257	355 251	6.8	94.1	352	1 : 30 000	129	10
21	1,7-Dimethyl-	340 266	347 258	7.5	76	w.s. ***	1 : 1 000	— †	3

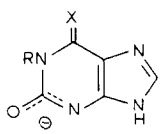
* N, neutral form; A, monoanion.

** The following compounds were not attacked by xanthine oxidase, 1 : 1000 during exposure of 96 h: 9-Methylxanthine and 3,7-dimethylxanthine; 9-methyl-6-thioxanthine and 3,7-dimethyl-6-thioxanthine.

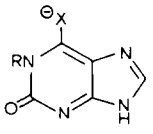
*** w.s., whole spectrum.

† Extremely slow reaction; the product was identified as 1,7-dimethyl-6-thiouric acid only on paper chromatograms.

Scheme 7.



12b: R = H; X = O
13b: R = Me; X = O
17b: R = H; X = S
18b: R = Me; X = S



12c: R = H; X = O
13c: R = Me; X = O
17c: R = H; X = S
18c: R = Me; X = S

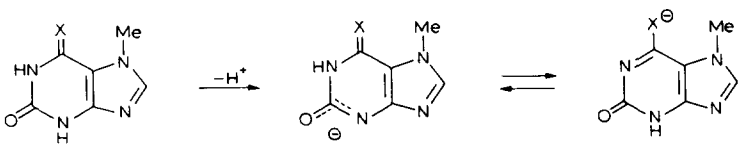
negative charge is spread only over the pyrimidine moiety, permitting attack of OH^- at position 8 in the imidazole ring. These considerations also explain why 3-methylxanthine (compound 14) is refractory.

The behavior of 6-thioxanthines may be explained by similar reasoning. The pK values of compound 17 and its 1-methyl derivative 18 are 6.2 and 6.7 respectively (Table III), i.e. at pH 8 they are present 98 and 95% as anions. Thus the latter most probably serve as substrates. In both compounds, the 3-NH group dissociates first and this process involves simultaneous tautomerisation to the 9-NH-forms [11], as in the xanthine series (scheme 7). Therefore the enhanced rate of oxidation of compound 18b,c may be explained along the same lines as for the anion of compound 13.

The oxidation of the 7-methyl derivatives 15 and 20 may be interpreted as follows. In the neutral forms, only the grouping (3)NH, (9)N is available for binding. In the anions, even this grouping is missing, unless the enzyme induces the tautomerisation compound 15a → compound 15b and compound 20a → compound 20b (scheme 8). The possible role of this process is supported by the observation that, in contrast to xanthine (compound 12) and 6-thioxanthine (compound 17), the rate of oxidation of compounds 15 and 20 is drastically reduced by introduction of 1-methyl substituents (see compounds 16 and 21 in Table III). Furthermore, the 3,7-dimethyl derivatives of compounds 12 and 17, in which the critical binding group is blocked, are refractory.

It should be noted that the 7-methyl substituent does not prevent oxidation at C-8, similar to our observation that a 3-methyl group permits enzymic attack at C-2 in compounds 2 and 6. Therefore the refractoriness of purines like 1-methylhypoxanthine cannot be explained simply by steric interference, although this factor may play a certain role.

Scheme 8.



15: X = O
20: X = S

15a: X = O
20a: X = S

15b: X = O
20b: X = S

(4) Purine-6,8-diones and 6-thioxopurin-8-ones (Table IV)

In the series of purine-6,8-diones, the 3-methyl derivative 23 was found refractory to attack by the new enzyme preparation, while previously we observed

TABLE IV

ENZYMIC OXIDATION OF PURINE-6,8-DIONES AND 6-THIOXOPURIN-8-ONES

No.	Compound	λ_{max} * (nm)		p*K* for anion formation	Anion at pH 8.0 (%)	Wave length used for measurement (nm)	Dilution of xanthine oxidase	Relative rate	Number of experiments
		N	A						
	A. Purine-6,8-diones **								
22	—	256	267	8.2	38.6	292	1 : 10 000	76	3
23	3-Methyl-	275	284	5.5	99.7	w.s. ***	1 : 3 000	—	2
24	7-Methyl-	258	270	7.8	61.3	300	1 : 10 000	287	4
25	3,7-Dimethyl-	277	285	6.0	99.0	w.s.	1 : 3 000	—	2
	B. 6-Thioxopurin-8-ones **								
11	—	332	310	7.1	88.8	300	1 : 15 000	94	3
		237	238						
26	3-Methyl-	341	338	5.0	99.9	w.s.	1 : 3 000	—	2
27	7-Methyl-	337	333	6.8	94.1	350	1 : 5 000	105	3
		237	246						

* N, Neutral form; A, monoanion.

** The following compounds were found to be refractory to xanthine oxidase, 1 : 1000, after 4 days exposure: 1- and 9-methylpurine-6,8-dione; 1- and 9-methyl-6-thioxopurin-8-one; 3,7-dimethyl-6-thioxopurin-8-one.

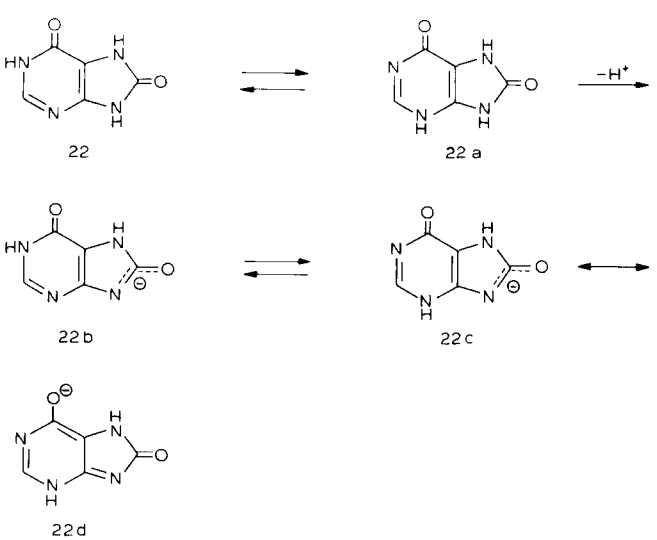
*** w.s., whole spectrum.

very slow oxidation by the enzyme [21]. On the other hand, the 7-methyl isomer 24 reacts nearly three times faster than the mother substance 22. This observation reminds one of the rapid oxidation of 1-methylxanthine (compound 13). It is therefore assumed that attachment of compound 22 via the grouping: (7)NH, (8)C = 0 inhibits the enzymic reaction to a certain degree. This view is supported by the observation that the rate of oxidation of purin-8-one is enhanced by introduction of a 7-Me substituent [1]. In the neutral form of compound 22, tautomerisation to the 3-NH form (compound 22a) similar to the "activation" of the hypoxanthine molecule, is difficult because of steric interference between 3- and 9-NH groups (see scheme 9) [10,11]. This obstacle can be overcome by anion formation at position 9 [12], (compound 22b—d). In fact, compound 22 (pK = 8.2) is present at pH 8 as a mixture of neutral forms and anions.

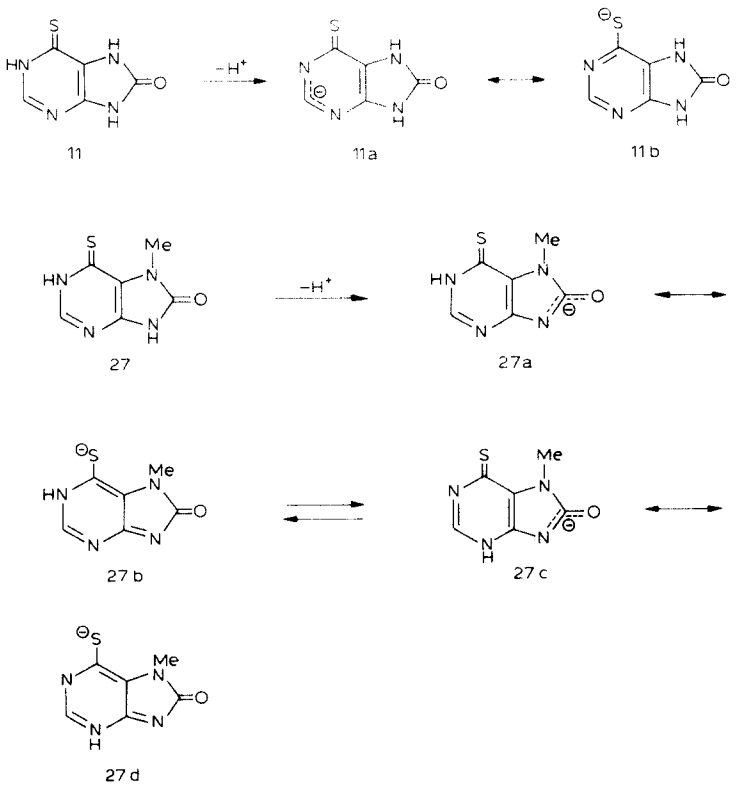
The 3,7-dimethyl homolog 25 was not attacked, although it is practically completely ionised at pH 8.

In the corresponding series of the 6-thioxopurin-8-ones, again only the mother substance 11 and its 7-methyl homolog 27 are attacked by xanthine oxidase, but the rate for compound 27 is only about half that of compound 11. At pH 8, both these purines are ionised to about 90% (Table I), but the NH-groups involved in monoanion formation are not identical [13]. In compound 11, the 1-NH dissociates first (scheme 10). The resulting anion is represented by the canonical forms 11a and b, the important contribution of form b being due to stabilisation by the aromatic structure of the pyrimidine ring. Therefore compound 11b may represent the "active form" that attaches itself to the enzymic center via (3)N, (9)NH. This is in accordance with the large hypsochromic shift of λ_{max} upon anion formation [13], but the enzyme may cause tautomerisation to a 3-NH form (like compound 27d in scheme 10, but 7-NH instead of 7-NMe). On the other hand, anion formation in compound 27 involves mainly

Scheme 9.



Scheme 10



9-NH [13]. In the anion 27a, resonance with compound 27b does not lead to an aromatic pyrimidine structure. Here the "active" forms may be compounds 27c–d, involving tautomerisation of the anion in the enzyme-substrate complex (scheme 10). Because of the different structures of the anions, direct comparison of the enzymic rates of compounds 11 and 27 is not possible.

Conclusions

The present experiments demonstrate that purines must bind to specific sites in the enzymic center of bovine milk xanthine oxidase in order to expose suitable C=N bonds of the substrate to those components of the enzyme molecule that mediate electron or hydride transfer [26]. All substrates used in this study are variants of hypoxanthine or xanthine, all have similar dimensions and thus presumably come into contact with the same parts of the active center, and in all of them either position 2 or 8 or both are available for oxidation. In those compounds which are attacked rapidly, the groupings (3)NH, (9)N or (3)N, (9)NH are necessary for proper binding. These groupings may be present in the ground state of the purines or may be created by suitable ionisation and/or tautomerisation processes, occurring during formation of the enzyme-substrate complex. When this critical binding site is blocked by *N*-methylation or when its formation by a tautomerisation process becomes impossible, the substrates are refractory or react at extremely low rates.

Auxiliary binding sites, such as (6)C = 0, (7)NH or the corresponding anionic groups, are supplied by amide (or thioamide) structures in the purine molecules. Some of these may enhance the affinity of the substrates; therefore *N*-methylation of such sites may inhibit the enzymic reaction. Other amide groups may impair proper binding by promoting complexation via unsuitable parts of the purine structure. In this case, *N*-methylation may accelerate enzymic oxidation.

A striking example is found in xanthines and 6-thioxanthines. Introduction of a 1-methyl substituent enhances the rate of oxidation of xanthine and 6-thioxanthine, but sharply reduces the rate of reaction of the 7-methyl derivatives 15 and 20. In compounds 12 and 17, 1-methylation prevents participation of the 1-NH- group in binding, this being an unsuitable mode of complexation. In the anions 15b and 20b, on the other hand, the same substitution at position 1 prevents tautomerisation to the 3-NH form which is necessary to create the proper binding site for these two purines. Furthermore, the 3,7-dimethyl derivatives of xanthine and 6-thioxanthine, in which anion formation fixes structures analogous to compounds 15b and 20b but which lack the binding site (3)NH, (9)N, are refractory.

We may conclude that in the active center of xanthine oxidase a particular site is responsible for attachment of a specific portion of the purines, serving as substrates. At present it cannot be decided whether this site belongs to the protein or to the flavin components of the enzyme. In this connection, it would be of great interest to determine whether deflavo-xanthine oxidase [28] shows the same or different substrate specificity as the complete enzyme, studied here.

Significant differences have been observed in the susceptibility of a number of purines to oxidation by milk xanthine oxidase of various degrees of purity. It is suggested that the inactive forms of the enzyme block certain groups in the enzymically active structure, which are required for proper binding of some substrates. Alternatively, the inactive forms may complex strongly with purine substrates and thus prevent their interaction with active enzyme. No answer to this problem is available at present.

References

1 Bergmann, F., Kwietny, H., Levin, G. and Brown, D.J. (1960) J. Am. Chem. Soc. 82, 598—605
2 Bergmann, F., Kleiner, M., Neiman, Z. and Rashi, M. (1964) Isr. J. Chem. 2, 185—196
3 Neiman, Z. and Bergmann, F. (1965) Isr. J. Chem. 3, 85—89
4 Neiman, Z. and Bergmann, F. (1965) Isr. J. Chem. 3, 161—172
5 Koppel, H.C. and Robins, R.K. (1958) J. Org. Chem. 23, 1457—1460
6 Bergmann, F. and Tamari, M. (1961) J. Chem. Soc. 4468—4472
7 Lichtenberg, D., Bergmann, F. and Neiman, Z. (1972) Isr. J. Chem. 10, 805—817
8 Bergmann, F. and Rahat, M. (1976) J. Chem. Soc. Perkin Trans. 1, in the press
9 Ross, L.O., Goodman, L. and Baker, B.R. (1959) J. Am. Chem. Soc. 81, 3108—3114
10 Lichtenberg, D., Bergmann, F. and Neiman, Z. (1971) J. Chem. Soc. (C) 1676—1682
11 Lichtenberg, D., Bergmann, F. and Neiman, Z. (1972) J. Chem. Soc. Perkin Trans. 1, 1676—1681
12 Rahat, M., Bergmann, F. and Tamir, I. (1974) J. Chem. Soc. Perkin Trans. 1, 2229—2236
13 Rahat, M., Bergmann, F. and Tamir, I. (1974) Isr. J. Chem. 12, 945—956
14 Bergmann, F. and Dikstein, S. (1955) J. Am. Chem. Soc. 77, 691—696
15 Dikstein, S. and Bergmann, F. (1955) J. Am. Chem. Soc. 77, 4671—4672
16 Bergmann, F., Burger-Rachamimov, H. and Tamari, M. (1963) Biochem. Biophys. Res. Commun. 12, 284—286
17 Levin, G., Kalmus, A. and Bergmann, F. (1960) J. Org. Chem. 25, 1752—1754
18 Hart, L.I., McGartoll, M.A., Chapman, H.R. and Bray, R.C. (1970) Biochem. J. 116, 851—864
19 Hart, L.I. and Bray, R.C. (1967) Biochim. Biophys. Acta 146, 611—613

20 Massey, V., Brumby, P.E., Komai, H. and Palmer, G. (1969) J. Biol. Chem. 244, 1682–1691
21 Bergmann, F., Levin, G., Kalmus, A. and Kwietny-Govrin, H. (1961) J. Org. Chem. 26, 1504–1508
22 Bergmann, F. and Dikstein, S. (1956) J. Biol. Chem. 223, 765–780
23 Bergmann, F. and Levene, L., in preparation
24 Bergmann, F. and Ungar, H. (1960) J. Am. Chem. Soc. 82, 3957–3960
25 Silberman, H.R. and Wyngaarden, J.B. (1961) Biochim. Biophys. Acta 47, 178–180
26 Massey, V., Edmondson, D., Palmer, G., Beacham, L.M. and Elion, G.B. (1971) Proc. Biochem. Soc Meet. 521, 10–11
27 Pfleiderer, W. and Nübel, G. (1961) Liebig's Ann. 647, 155–166
28 Komai, H., Massey, V. and Palmer, G. (1969) J. Biol. Chem. 244, 1692–1700