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The Oxidation of 4-Pyrimidinone and 4-Quinazolinone and Their *N*-Methyl Derivatives by Milk Xanthine Oxidase

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4-Pyrimidinone, 4-quinazolinone, and each of their 1-methyl derivatives are oxidized to the corresponding 2,4-diones by milk xanthine oxidase. Steady-state kinetic parameters have been evaluated for the enzymatic oxidation of these substrates over the pH range 5.0–10.5. The pH dependences of k_c/K_m for each of 4-pyrimidinone and 4-quinazolinone are consistent with the neutral molecules of these species being substrates, but their anionic conjugate bases not being enzymatically oxidized. Apart from this substrate ionization, k_c and K_m do not show any dramatic pH dependence. 1-Ethyl-4-pyrimidinone is slowly oxidized by this enzyme, and 3-methyl-4-pyrimidinone is an extremely poor substrate; 3-methyl-4-quinazolinone is not enzymatically oxidized. These latter two species are competitive inhibitors for the oxidation of 4-pyrimidinone. The 2- and 4-pyridinones, the 2- and 4-quinolinones, 1-isoquinolinone, and each of their *N*-methyl derivatives have been shown to be reversible inhibitors for this enzyme and I_{50} values have been evaluated. These data are shown to be consistent with the neutral 1H tautomers of each of 4-pyrimidinone and 4-quinazolinone being the true substrates for this enzyme. The low reactivity of 4-quinazolinone as a substrate can probably be traced to reversible inhibition by 4(3H)-quinazolinone of the enzymatic oxidation of 4(1H)-quinazolinone.

Milk xanthine oxidase catalyzes the oxidation of a wide variety of hydroxylated purines, pteridines, and related heterocyclic species (*1–6*). Most such substrates exist in aqueous solution as rapidly equilibrating mixtures of a number of keto and enol tautomers, with one or more keto tautomers usually being the major species present. The question of which of these tautomers is the actual substrate species that binds to the enzyme to produce the productive Michaelis complex has been addressed on a number of occasions (*1, 4–6*). The usual approach to this problem involves the investigation of the various *N*-methyl derivatives as substrates for the enzymatic oxidation, and comparison of the relative reactivities of each of these species with that of the parent hydroxylated heterocycle. Further information may also be gained from studies of the regiospecificity of the enzymatic oxidation in the various *N*-methylated derivatives of certain purines and pteridines which contain multiple sites that are susceptible to oxidation.

Unfortunately, there is an inherent uncertainty in the interpretation of the results of all such studies. Thus, if a particular *N*-methyl derivative is found to be either a nonsubstrate or a poor substrate, it is not clear whether this observation

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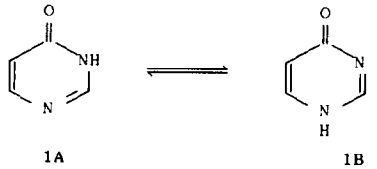
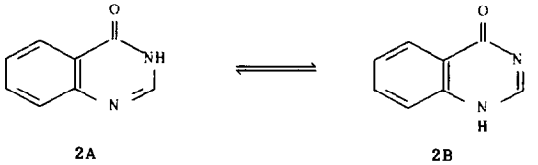
should be interpreted in terms of the wrong "tautomeric form" being presented to the enzyme or in terms of a simple steric effect arising from the replacement of an NH unit by the larger NCH_3 moiety. A further complication lies in the fact that many hydroxylated substrates are involved in one or more acid–base equilibria in the broad pH range over which this enzyme is active. The question then arises as to whether both conjugate acid and conjugate base are acceptable substrates. The answer to this question is further complicated by the observation that depending upon the substituent present, deprotonation of purine derivatives may occur preferentially on either the pyrimidine or imidazole rings (*7, 8*).

Previous reports (*9–11*) from our laboratory have explored the specificity of milk xanthine oxidase for the oxidation of cationic heterocyclic substrates. Such substrates exist as unique species in aqueous solution, since they are susceptible to neither tautomerization nor acid–base equilibration in the pH region within which the enzyme is active. Several important enzyme–substrate binding interactions have been identified, and a partial mapping is now available of the substrate binding region of this enzyme (*9, 11*). We have shown (*11*) that this active site map allows the ready rationalization of the observed specificity of the enzymatic oxidation of purine to hypoxanthine to xanthine to uric acid, and also predicts the observed susceptibility of pteridine derivatives to enzymatic oxidation in both the pyrimidine and pyrazine rings.

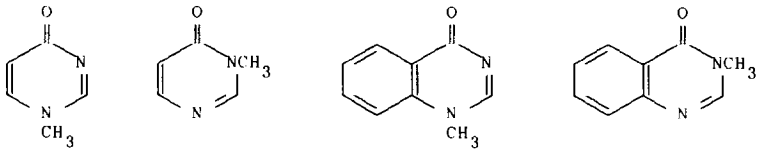
Further elaboration of this active site region should ultimately allow the prediction of the enzymatic specificity for various tautomers of the hydroxylated purines and related species. This goal requires the systematic quantitative study of substrate specificity, as well as the investigation of reversible inhibition for all potential substrates that are found to be inactive. Such a systematic study will be most effective if it commences with potential substrates that have a minimum number of tautomeric possibilities and are involved in only simple acid–base equilibria. As an initial step in this direction, we now report our investigation of the oxidation of 4-pyrimidinone (**1A** $\rightleftharpoons$ **1B**) (4-hydroxypyrimidine) and 4-quinazolinone (**2A** $\rightleftharpoons$ **2B**) (4-hydroxyquinazoline) and each of their 1-methyl and 3-methyl derivatives (**3–6**) by milk xanthine oxidase and, also, the pH dependence of the kinetic parameters for these reactions.



1A 1B



2A 2B



3 4 5 6

EXPERIMENTAL DETAILS

Substrates and inhibitors. 4(3H)-Pyrimidone, 4-hydroxyquinazoline, 2-hydroxypyridine, 4-hydroxypyridine, 1-methyl-2-pyridone, 2-hydroxyquinoline, 4-hydroxyquinoline, and isocarbostyril were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. 1-Methyl-4-pyridinone (*12*), 1-methyl-2-quinolinone, 1-methyl-4-quinolinone (*13*), 2-methyl-1-isoquinolinone, and 3-methyl-4-quinazolinone (**6**) (*14*) were prepared by treatment of a basic ethanolic solution of the appropriate unmethylated heterocycle with methyl iodide. 1-Methyl-4-quinazolinone (**5**) was prepared by the method of Scarborough and Minielli (*15*) via aminolysis of *N*-methylisatoic anhydride (Aldrich) to 2-methylaminobenzamide and then ring closure in formic acid. Methylation of 4-pyrimidinone with methyl iodide in basic ethanol produces a mixture of 1-methyl-4-pyrimidinone (**3**) and 3-methyl-4-pyrimidinone (**4**) (*16*), which can be conveniently separated by column chromatography on alumina upon elution with chloroform (3-methyl derivative) and 1 : 1 chloroform–ethanol (1-methyl isomer). 1-Ethyl-4-pyrimidinone was similarly prepared. This latter product is extremely hygroscopic, but the initial oily product was found to crystallize in a vacuum desiccator over phosphorous pentoxide. All commercial and synthetic products were recrystallized to constant melting points consistent with literature data, and their identities were confirmed by PMR spectroscopy.

Enzyme solutions. Stock solutions of xanthine oxidase from buttermilk were prepared and assayed as described previously (*9*) from salicylate-stabilized suspensions of Grade III enzyme purchased from Sigma Chemical Company, St. Louis, Missouri.

Kinetic studies. Steady-state initial velocities of the enzyme-catalyzed oxidation of each substrate were measured at 25°C in buffers of ionic strength 0.1 in the presence of potassium ferricyanide (0.4 mM) as electron acceptor. All reactions were monitored spectrophotometrically by monitoring ferricyanide ion reduction at 420 nm using a Varian Cary 210 spectrophotometer. None of the present substrates exhibited any significant nonenzymatic oxidation by ferricyanide ion under the current reaction conditions.

Initial velocities were measured at 10 or more substrate concentrations which spanned the K_m value at each pH. After checking visually for linearity in Lineweaver–Burk plots, the kinetic parameters k_c and K_m were evaluated by fitting the data directly to the Michaelis–Menten equation by a computer-based regression analysis based upon the Marquardt algorithm. Substrate inhibition was not observed for any of the substrates investigated in the current study.

Reversible inhibition was initially investigated by evaluating the initial rate of the enzyme-catalyzed oxidation of 4-pyrimidinone (0.036 mM) at pH 8.5 as a function of the concentration of the potential inhibitor species. Inhibitor concentrations (I_{50}) producing 50% inhibition under these conditions were evaluated from linear $1/v$ versus [I] plots. In more detailed inhibition studies, K_i values were evaluated from steady-state kinetic studies at three or more different inhibitor concentrations.

Product studies. A solution of the potential substrate, saturated with oxygen as electron acceptor, was treated with an aliquot of concentrated enzyme solution. Reaction progress was monitored spectrophotometrically until no further significant spectral change was observed upon addition of a further aliquot of enzyme solution. For very slow reactions, daily additions of additional enzyme solution were made to counter inactivation of the enzyme through denaturation.

RESULTS

4-Pyrimidinone and 4-quinazolinone have each been previously shown to be substrates for milk xanthine oxidase in neutral aqueous solutions (*2, 17, 18*). While 2,4-quinazolinedione (2,4-dihydroxyquinazoline) was established as the enzymatic oxidation product from 4-quinazolinone (*18*), the oxidation product from 4-pyrimidinone does not seem to have been investigated. There has been no investigation of the pH dependence of the enzymatic oxidation of either of these substrates, and there seem to be no reports of any of the *N*-methyl derivatives of these two heterocycles having been investigated as potential substrates or inhibitors.

The time dependence of the electronic absorption spectrum of 4-pyrimidinone in the presence of milk xanthine oxidase in aqueous solution at pH 7.5 was followed until addition of further enzyme produced no further spectral change. The spectrum of the oxidation product at pH 7.5 displayed λ_{max} (ε) = 259 nm (7500 M^{-1} cm^{-1}), which is essentially identical to that of uracil under similar conditions [259.5 nm (8200 M^{-1} cm^{-1}) (*19*)]. Adjusting this product solution to pH 14 produced a shift in λ_{max} to 285 nm as is typical of uracil at this pH (*19*). These observations establish that enzymatic oxidation of this molecule occurs preferentially at C-2 to produce uracil (2,4-pyrimidinedione).

The rates of the enzymatic oxidation of each of 4-pyrimidinone and 4-quinazolinone were examined as a function of substrate concentration for the range pH 5.0 to 10.5. In all cases, kinetic saturation consistent with the Michaelis–Menten equation was observed. The pH dependences of the individual parameters, k_c and K_m, are indicated in Table 1. While no simple pH dependence is ascertainable for these individual parameters, the pH dependence of the specificity constant (k_c/K_m) is quite informative (Fig. 1). For each of these substrates, k_c/K_m is pH independent in neutral solution, but decreases at high pH in a manner consistent with the equation

$$k_c/K_m = \frac{\alpha}{1 + K_a/[H^+]}. \qquad [1]$$

TABLE 1

pH DEPENDENCE OF k_c AND K_m FOR 4-QUINAZOLINONE AND 4-PYRIMIDINONE

pH	k_c (s^{-1})	K_m (mM)	k_c/K_m ($M^{-1}\ s^{-1}$)
	4-Quinazolinone		
5.2	0.078 ± 0.008	0.007 ± 0.001	1.1×10^4
6.2	0.093 ± 0.009	0.015 ± 0.002	6.2×10^3
7.1	0.11 ± 0.01	0.018 ± 0.003	6.1×10^3
8.0	0.33 ± 0.03	0.039 ± 0.002	8.5×10^3
8.7	0.40 ± 0.04	0.035 ± 0.004	1.1×10^4
10.0	0.34 ± 0.03	0.17 ± 0.01	2.0×10^3
10.4	0.32 ± 0.03	0.33 ± 0.03	1.0×10^3
	4-Pyrimidinone		
5.0	6.0 ± 0.5	0.011 ± 0.005	5.5×10^5
6.0	4.5 ± 0.5	0.0065 ± 0.0008	6.9×10^5
6.5	7.5 ± 0.1	0.007 ± 0.001	1.1×10^6
7.7	8.3 ± 0.2	0.012 ± 0.002	6.9×10^5
8.5	10.9 ± 0.5	0.017 ± 0.003	6.4×10^5
9.4	22 ± 2	0.18 ± 0.02	1.2×10^5
10.0	28 ± 3	0.50 ± 0.05	5.6×10^4
10.5	50 ± 5	3.3 ± 0.5	1.6×10^4

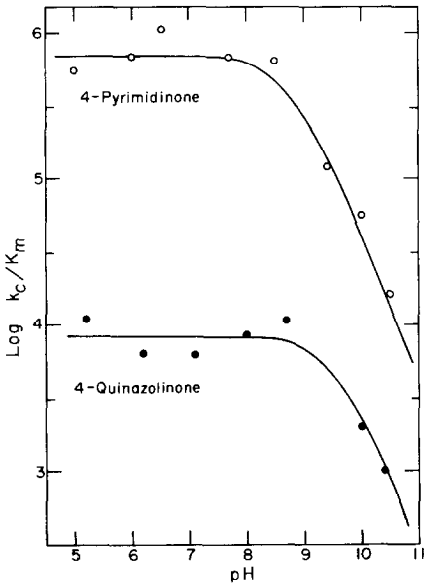


FIG. 1. The pH dependence of $\log(k_c/K_m)$ for the oxidation of 4-pyrimidinone and 4-quinazolinone by milk xanthine oxidase. Curves are drawn using Eq. [1] and the parameters in the text.

The curves in Fig. 1 correspond to Eq. [1], with $\alpha = 7 \times 10^5$ liters/mol/s and $pK_a = 8.8$ for 4-pyrimidinone, and $\alpha = 9 \times 10^3$ liters/mol/s and $pK_a = 9.7$ for 4-quinazolinone.

1-Methyl-4-pyrimidinone (**3**) and 1-methyl-4-quinazolinone (**5**) also proved to be readily oxidized by milk xanthine oxidase, although these two substrates had significantly higher K_m values than the corresponding unmethylated species. The pH dependences of the kinetic parameters for these two substrates are listed in Table 2. Again there is no simple pH dependence for the individual kinetic parameters; however, k_c/K_m appears to be pH independent for 1-methyl-4-quinazolinone, and to show a slight increase with pH in the case of 1-methyl-4-pyrimidinone (Table 2). The oxidation product of 1-methyl-4-pyrimidinone displayed λ_{max} (ε) = 266 nm (8250 M^{-1} cm^{-1}), consistent with the formation of 1-methyluracil upon oxidation at C-2 [λ_{max} (ε) = 266 (9500) (*20*)].

1-Ethyl-4-pyrimidinone proved to be a much poorer substrate than its 1-methyl homolog. Data for this species at pH 8.5 are also included in Table 2.

A very slow reaction was noted when 3-methyl-4-pyrimidinone (**4**) was tested as a substrate for milk xanthine oxidase. After 5 days in the presence of 4×10^{-7} M enzyme (replenished daily) at pH 7.5 and 25°C, the absorption spectrum of a 0.2 mM solution of 3-methyl-4-pyrimidinone displayed λ_{max} (ε) = 258 nm (6030 M^{-1} cm^{-1}), which is similar to that reported (*20*) for 3-methyluracil [258 nm (7943 M^{-1} cm^{-1})]; however, this species was such a poor substrate that no convincing kinetic data were obtainable. We conclude that this 3-methyl derivative is an

TABLE 2

pH DEPENDENCE OF k_c AND K_m FOR 1-METHYL-4-QUINAZOLINONE, 1-METHYL-4-PYRIMIDINONE, AND 1-ETHYL-4-PYRIMIDINONE

pH	k_c (s^{-1})	K_m (mM)	k_c/K_m (M^{-1} s^{-1})
	1-Methyl-4-quinazolinone		
6.0	0.91 ± 0.07	1.1 ± 0.1	800
7.0	2.2 ± 0.2	2.2 ± 0.1	1000
7.5	3.8 ± 0.5	4.3 ± 0.9	900
8.0	6.0 ± 0.5	5.3 ± 0.1	1100
9.0	6.7 ± 0.7	6.2 ± 0.7	1100
9.5	4.0 ± 0.3	3.8 ± 0.3	1050
10.0	4.1 ± 0.3	4.1 ± 0.3	1000
10.5	3.7 ± 0.3	2.2 ± 0.2	1700
	1-Methyl-4-pyrimidinone		
7.0	4.8 ± 0.3	44 ± 4	110
8.0	8 ± 1	47 ± 6	170
8.5	6.1 ± 0.4	34 ± 3	180
9.0	5.8 ± 0.3	28 ± 1	210
10.0	12 ± 1	48 ± 6	260
	1-Ethyl-4-pyrimidinone		
8.5	0.50 ± 0.05	280 ± 50	1.8

extremely poor substrate and estimate that it has a specificity constant at least 10 times smaller than that reported for the 1-ethyl derivative in Table 2. 3-Methyl-4-pyrimidinone acted as a reversible competitive inhibitor for the oxidation of 4-pyrimidinone, and displayed K_i = 74 mM at pH 8.5.

No enzymatic oxidation of 3-methyl-4-quinazolinone (**6**) was observed even after prolonged incubation with 4×10^{-7} M enzyme. This species was a reversible competitive inhibitor for the oxidation of 4-pyrimidinone with K_i = 4.1 mM at pH 8.5.

We have also briefly surveyed 2- and 4-pyridinone, 2- and 4-quinolinone, 1-isoquinolinone, and the *N*-methyl derivatives of these heterocycles as reversible inhibitors of the oxidation of 4-pyrimidinone by xanthine oxidase at pH 8.5. All of these species inhibited this enzymatic oxidation (Table 3). 4-Quinolinone was selected for a more detailed study of this inhibition. This latter species proved to be a competitive inhibitor of the oxidation of 4-pyrimidinone, with K_i = 0.5 and 0.7 mM at pH 8.0 and 10.0, respectively. 4-Quinolinone was also established as a competitive inhibitor of the enzymatic oxidation of the *N*-methylquinolinium cation, with K_i = 1 mM at pH 10.5.

DISCUSSION

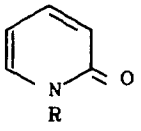
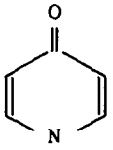
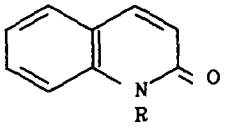
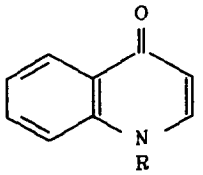
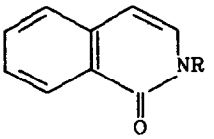
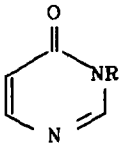
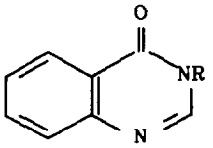
4-Pyrimidinone and 4-quinazolinone are each oxidized at C-2 by milk xanthine oxidase to give 2,4-pyrimidinedione (uracil) and 2,4-quinazolinedione, respectively. The 1-methyl derivatives of each of 4-pyrimidinone and 4-quinazolinone are also readily enzymatically oxidized at C-2. 3-Methyl-4-quinazolinone is not enzymatically oxidized, while 3-methyl-4-pyrimidinone appears to be an extremely poor substrate for this enzyme. However, both of these 3-methyl derivatives competitively inhibit the enzymatic oxidation of 4-pyrimidinone.

The pH dependences of k_c/K_m for 4-pyrimidinone and 4-quinazolinone (Fig. 1) reflect pK_a values that are quite similar to the reported pK_a values for deprotonation of these substrates [8.6 and 9.8, respectively (*21, 22*)]. These pH profiles are only readily interpretable in terms of the neutral molecules being the preferred substrates for this enzyme, with the α parameter of Eq. [1] being the specificity constant for the neutral species. The anionic conjugate bases do not appear to bind at the active site, although we cannot rule out their binding several orders of magnitude less tightly than the neutral molecules. This lack of (or very low) enzymatic activity toward the anionic species is not surprising in view of the fact that cationic substrates are readily oxidized by this enzyme in basic solutions (*9–11*). The ready oxidation of the neutral 1-methyl derivatives of 4-pyrimidinone and 4-quinazolinone is also consistent with the neutral species of the parent compounds being the preferred substrates.

This preference for the neutral rather than the anionic species of these heterocycles as substrates for this enzyme contrasts with the general impression one obtains from the xanthine oxidase literature regarding enzymatic specificity for hydroxylated purines and related species. The literature contains numerous comments to the effect that anionic purine derivatives are acceptable substrate species

TABLE 3

REVERSIBLE INHIBITION OF XANTHINE OXIDASE CATALYSIS[a]

Inhibitor	I_{50} (mM) R = H	I_{50} (mM) R = CH_3
	48	200
	15	49
	≈2[b]	4.3
	2.1	11
	1.5	>1[b]
	[c]	320
	[c]	14

[a] For the oxidation of 0.036 mM 4-pyrimidinone, at pH 8.5

[b] Limited solubility.

[c] In equilibrium with the 1H tautomer, which is a substrate.

[e.g., (*4, 5, 23, 24*)]; however, there have been few detailed studies of the pH dependence of xanthine oxidase catalysis. Most pH–rate profiles have been determined at a single substrate concentration and are thus complex functions of the true pH dependence of k_c and K_m as well as any contributions from substrate pK_a

values. Many purine-derived substrates also show pronounced substrate inhibition with this enzyme, and thus studies of pH dependence at a single substrate concentration, as are often reported, may also be influenced by the pH dependence of this substrate inhibition. In this regard it should be noted that substrate inhibition of hypoxanthine oxidation becomes less important at higher pH than in neutral solution (*25*). If this is typical of all substrates, then it is possible that the observed activity in basic solutions is due not to the reactivity of substrate anions, but rather to decreasing substrate inhibition of the reaction of the neutral substrate molecules.

Much of the confusion regarding substrate anions as suitable substrates for this enzyme seems to stem from the early report on the pH dependence of K_m for xanthine (*26*). This pronounced pH dependence was interpreted in terms of the xanthine monoanion being the true substrate, whereas the neutral molecule and dianion were assumed not to be bound in the active site. This interpretation was subsequently accepted (*23, 27*), but it appears to rely entirely upon the observation that log K_m is approximately linear in pH when K_m is expressed in terms of the concentration of the monoanion alone. It is not at all clear why this linearity should be taken as proof that the monoanion is the actual substrate. In fact, if the reported (*26*) experimental K_m is expressed in terms of the concentration of the neutral xanthine molecule alone, then K_m for neutral xanthine varies little in the range pH 4.4–10.5 which is similar to the observations of only weakly pH-dependent K_m values for substrates which exist solely as neutral molecules throughout this pH range (e.g., Table 2).

The general drift of turnover numbers to higher values with increasing pH in Tables 1 and 2 is also found for xanthine (*27*) and other substrates (*28*). The general lack of any strong dependence upon pH for either k_c or K_m (when expressed in terms of the concentration of the substrate neutral molecule) for any xanthine oxidase substrate clearly indicates that the activity of this enzyme is not controlled by any simple acid–base equilibria of enzymatic functional groups. This is not surprising in view of the complex nature of the k_c and K_m parameters that are deduced for the complicated kinetic scheme that has been found necessary to describe the enzymatic activity (*29, 30*).

It has been briefly reported (*2*) that the 4-hydroxy derivatives of pyrido[2.3-*ld*]pyrimidine and pyrido[3.2-*d*]pyrimidine, both of which presumably exist predominantly as their 4-oxo tautomers in aqueous solution, are oxidized at C-2 by xanthine oxidase. However, the most detailed studies of close analogs of the 4-pyrimidinones and 4-quinazolinones of the current study can be found in the investigations of the enzymatic oxidation of 4-pteridinone, hypoxanthine, allopurinol, and their *N*-methyl derivatives. Products of the enzymatic oxidation of these substrates are summarized in Table 4. Surprisingly, detailed analyses of the pH dependences of the enzymatic oxidation do not seem to be available for any of these extensively studied substrates.

Hypoxanthine (6-hydroxypurine) is the closest purine analog of the substrates of Table 1 of the current study. This well-studied substrate is enzymatically oxidized to xanthine (*1, 2, 4*) which is exactly analogous to the regiospecificity of the enzymatic oxidation of 4-pyrimidinone and 4-quinazolinone. There does not seem

to have been a detailed study of the pH dependence of the enzymatic oxidation of hypoxanthine,[2] although this substrate is reported to display increasing K_m with increasing pH in the region pH 7.8–10.8 (*28*). This is similar to the current observations for the two substrates in Table 1, and so is also suggestive of an enzymatic preference for the neutral molecule over the hypoxanthine anion. However, the

[2] Springer and co-workers report (*31*) that the relative rates of hypoxanthine oxidation at a single substrate concentration pass through a maximum in the vicinity of pH 8.2.

TABLE 4

PRODUCTS FROM REACTIONS CATALYZED BY XANTHINE OXIDASE

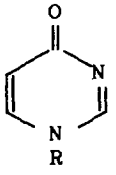
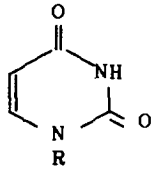
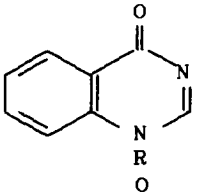
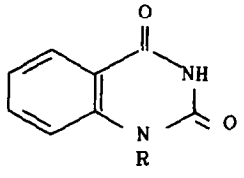
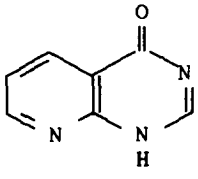
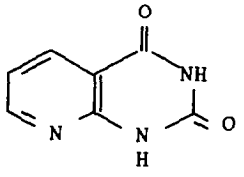
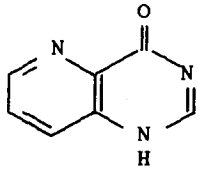
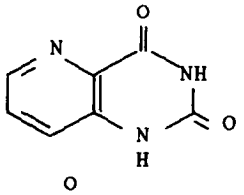
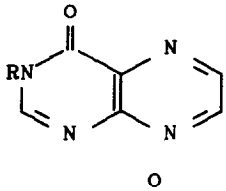
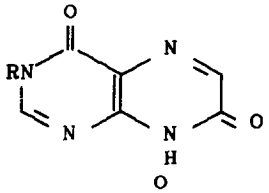
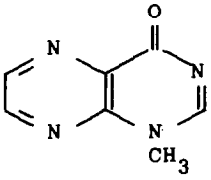
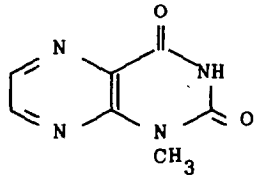
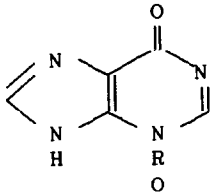
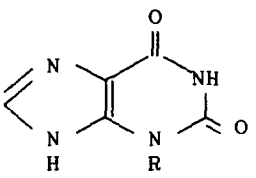
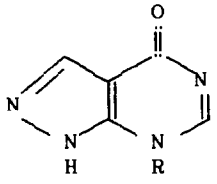
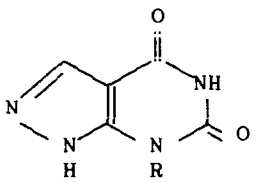
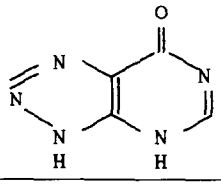
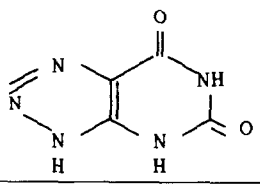
Substrate	R	Product	Reference
	H, CH_3		[a]
	H, CH_3		(*18*)[a]
			(*2*)
			(*2*)
	H, CH_3		(*5*)
			(*5*)

TABLE 4—*Continued*

Substrate	R	Product	Reference
	H, CH_3		(*1, 2, 4*)
	H, CH_3		(*2, 32, 33*)
			(*34*)

[a] Present work.

predominant anion formed from hypoxanthine [pK_a 8.4 (*4*)] arises from deprotonation of the imidazole ring, not from deprotonation of the pyrimidinone moiety (*7*). Thus, comparison of the enzymatic oxidation of hypoxanthine in basic solutions with that of 4-pyrimidinone and 4-quinazolinone does not permit an exact analogy to be drawn.

3-Methylhypoxanthine is reported to be slowly oxidized at C-2 at pH 8.0 (*1, 2, 4*), whereas its 1-methyl isomer is reported to be a reasonable substrate at pH 6.8 (*2*) (although the oxidation product does not appear to have been established) but a nonsubstrate at pH 8.0 (*1, 4*). Thus at pH 8.0 these two isomers show the same order of relative reactivities toward enzymatic oxidation as those that are observed for the 1-methyl and 3-methyl derivatives of 4-pyrimidinone and 4-quinazolinone.[3]

4-Pteridinone undergoes enzymatic oxidation at C-7 rather than at C-2 (*5*), at rates which decrease dramatically above pH 8.0 and so are consistent with a much lower reactivity of the anion than the neutral molecule. The difference in regiospecifity of the enzymatic oxidation of 4-pteridinone and 4-quinazolinone does not permit any direct comparison of these two species as substrates. However, 1-methyl-4-pteridinone is oxidized at a significant rate at C-2 (*5*), in a similar manner to the 1-methyl derivatives of 4-pyrimidinone and 4-quinazolinone. Furthermore,

[3] Note that the numbering of the atoms of the purine ring system results in N-1 and N-3 of hypoxanthine being equivalent to N-3 and N-1, respectively, of the pyrimidine and quinazoline derivatives. Similarly, the numbering of the atoms of the pyrazolo[3.4-*d*]pyrimidine ring system results in N-5, C-6, and N-7 of allopurinol and its derivatives being equivalent to N-3, C-2, and N-1, respectively, of the pyrimidine and quinazoline derivatives.

3-methyl-4-pteridinone is an extremely poor substrate and is specifically oxidized at C-7 rather than at C-2 (*5*); this observation is consistent with 3-methyl-4-quinazolinone not being oxidized by this enzyme.

Allopurinol (the keto tautomer of 4-hydroxypyrazolo[3,4-*d*]pyrimidine), an isomer of hypoxanthine, is enzymatically oxidized at C-6 (*2, 32*), as is its 7-methyl derivative (*33*). The 5-methyl derivative, however, is not attacked by this enzyme (*33*). Thus the analogy with the corresponding 4-quinazolinone derivatives is exact.[3] 6-Hydroxy-8-azapurine (8-azahypoxanthine) has also been established to undergo oxidation at C-2 by milk xanthine oxidase (*34*).

The overall picture that emerges for all of these 4-pyrimidinone analogs and their *N*-methyl derivatives is quite consistent. In all cases, the 1-methyl derivatives (or their equivalents in other ring-numbering systems) are poorer substrates than their unmethylated analogs, and in all cases enzymatic oxidation of these 1-methyl derivatives occurs at C-2 (or its equivalent). In all cases, the 3-methyl derivative (or its equivalent) is a much poorer substrate than its 1-methyl isomer, with no enzymatic oxidation being observed for the 3-methyl derivatives in some cases. In all cases, rates of enzymatic hydrolysis decrease in basic solutions, consistent with the neutral molecules rather than the anions being the favored substrates for the enzyme.

The observation that the presence of a 1-methyl substituent in 4-pteridinone induces enzymatic oxidation at C-2 and overrides the pronounced preference for C-7 oxidation in this heterocyclic system (*5*) is a strong argument in favor of the 1H-4-oxo tautomers being the preferred enzymatic substrates for C-2 oxidation in the tautomeric mixtures of these 4-pyrimidinone derivatives. This conclusion is supported by the observation that in all cases the 1-methyl-4-oxo derivatives (or their equivalents) are reasonable substrates, whereas the corresponding 3-methyl-4-oxo isomers (or their equivalents) are very poor substrates or nonsubstrates. This latter fact, by itself, could be simply construed as a simple steric hindrance toward the binding of the 3-methyl substituent. However, when considered in conjunction with the other factors cited herein, this observation is most simply reconciled with an enzymatic preference for the electronic density distribution associated with the 1H-4-oxo (and 1-methyl-4-oxo) species, when enzymatic oxidation occurs at C-2.

This conclusion indicates that the minor of the two keto tautomers is the actual substrate in the enzymatic oxidation of 4-pyrimidinone and 4-quinazolinone. These two compounds each exist predominantly as a rapidly equilibrating mixture of their keto tautomers in neutral aqueous solutions. In both cases the 3H tautomer predominates over the 1H tautomer, with the ratio being about 2 : 1 in favor of the 3H tautomer for 4-pyrimidinone (*35*) and 10 : 1 in the case of 4-quinazolinone (*36*). In neither case is there any significant concentration of the 4-hydroxy tautomer present in the equilibrium mixture.

This enzymatic preference for the minor tautomer also allows the rationalization of the fact that 4-quinazolinone appears to be a much poorer substrate than 4-pyrimidinone (Table 1), whereas 1-methyl-4-quinazolinone is a significantly better substrate than its 1-methyl-4-pyrimidinone analog (Table 2). The greater reactivity of the enzyme toward quinazoline rather than pyrimidine derivatives is expected

in view of the role that has been established for hydrophobic interactions between enzyme and substrate (*9, 11*). Thus, it is the reduced reactivity of 4-quinazolinone relative to 4-pyrimidinone that is surprising. Some of this low reactivity can be accounted for in terms of the actual substrate being a species that is present at only about 10% of the concentration of the total tautomeric mixture. However, this fact alone is not sufficient to account for the much lower reactivity of 4-quinazolinone than of 4-pyrimidinone in these enzymatic oxidations.

We suggest that the much lower apparent reactivity of 4-quinazolinone as a substrate for this enzyme can be traced to the fact that not only is the major 3H tautomer (**2A**) not a substate; it is actually a reversible inhibitor of the enzymatic oxidation of the minor 1H tautomer (**2B**). In support of this contention is our observation that 3-methyl-4-quinazolinone is a significant competitive inhibitor of the enzymatic oxidation of other substrates, and that in general such *N*-methyl heterocycles are actually poorer inhibitors than the corresponding NH species (Table 3). The simplest steady-state rate equation for a reaction subject to competitive inhibition is

$$\frac{v}{[E]} = \frac{k_c}{1 + K_m(1 + [I]/K_i)/[S]}. \qquad [2]$$

For the case of a substrate and an inhibitor that are rapidly equilibrating tautomers, $[I] = a[S]$, so that Eq. [2] may be transformed as in

$$\frac{v}{[E]} = \frac{k_c/(1 + aK_m/K_i)}{1 + K_m/\{[S]\,(1 + aK_m/K_i)\}} \qquad [3]$$

$$= \frac{k_c/(1 + aK_m/K_i)}{1 + K_m(1 + a)/\{[T]\,(1 + aK_m/K_i)\}} \qquad [4]$$

where $[T] = [I] + [S]$. Equation [4] predicts that the experimentally measured turnover number will be $k_c/(1 + aK_m/K_i)$, which is of course less than the true k_c for substrate. It should be noted that the experimentally measured k_c value for 4-quinazolinone is unusually small in comparison to the value usually found for good substrates for this enzyme; for instance, the values of k_c for 4-pyrimidinone in Table 1 and for the 1-methyl derivatives in Table 2 are similar in magnitude to those found for typical purine derived substrates [e.g., 14 s^{-1} for xanthine at pH 8.5 (*37*) and 17 s^{-1} for hypoxanthine, i.e., 121% of the value for xanthine (*6, 24*)].

Inhibition by 4(3H)-quinazolinone and its 3-methyl derivative implies the availability of a nonproductive binding mode for these molecules in which C-2 is oriented away from the catalytically active functional groups of the enzyme. Since these 4-quinazolinone derivatives are almost isosteric with the analogous 4-pteridinone species, an obvious nonproductive binding mode is available. These 4-pteridinone species bind so that C-7 is correctly oriented for enzymatic attack. Such an orientation has been shown to be consistent with a crucial hydrogen bonding interaction in the active site of the enzyme (*9, 11*). We suggest that the 4(3H)-quinazolinone tautomer is predominantly bound in an orientation analogous to the productive orientation for C-7 oxidation of 4(3H)-pteridinone and its 3-methyl derivative (see Fig. 2). Of course, enzymatic oxidation would not be

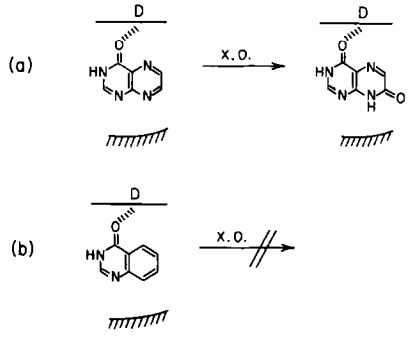


FIG. 2. Schematic representation of the binding of (a) 4(3H)-pteridinone as a substrate susceptible to oxidation at C-7, and (b) 4(3H)-quinazolinone as a competitive inhibitor, using the active site map deduced previously (*9, 11*). Species D is an enzymatic hydrogen bond donor, and the hatched area represents a region of steric congestion.

expected to occur at the electronically unactivated C-7 atom of a quinazoline derivative.

Analogous considerations regarding the minor tautomer as substrate and the major tautomer as inhibitor are expected also to apply to 4-pyrimidinone. However, in this case the difference in the relative concentrations of minor and major tautomers is much less than for 4-quinazolinone (*35, 36*); furthermore, 4(3H)-pyrimidinone is expected to be a much poorer inhibitor than is its quinazolinone analog since the K_i for 3-methyl-4-quinazolinone (4.1 mM) is approximately 20-fold smaller than that for 3-methyl-4-pyrimidinone (74 mM).

The possibility that minor tautomeric species of the substrate may play a role in the reaction mechanism of these enzymatic oxidations has been recognized for some time. However, this possibility has usually been discussed (*4–6, 24, 33*) in terms of very speculative enzyme-catalyzed tautomerism, deprotonation, and mesomerism (!) of the predominant substrate species. In actual fact, there seems to be no evidence requiring such enzyme-catalyzed processes. Rather, earlier extensive studies of substrate specificity should be reevaluated in the light of the likelihood that (i) substrate anions are not active substrates, and (ii) major tautomers are potential reversible inhibitors of enzymatic activity toward minor tautomers. In many cases, such reevaluation will require more extensive and systematic kinetic data, as a function of both substrate structure and pH, than are currently available.

The dramatically lower catalytic activity observed toward 1-ethyl-4-pyrimidinone relative to its 1-methyl homolog is similar to that previously reported (*9*) for corresponding *N*-methyl and *N*-ethyl heterocyclic cations as substrates for this enzyme. The value of $(k_c/K_m)^{Me}/(k_c/K_m)^{Et} = 100$ from the data in Table 2 is similar in magnitude to the values of 32 and 36 found (*9*) for this specificity constant ratio for quinolinium and nicotinamide cations, respectively. This observation, in conjunction with the similar competitive inhibition demonstrated in this work toward 4-pyrimidinone and the *N*-methylquinolinium cation as substrates, and the previously reported (*10*) similarity in the deuterium kinetic isotope effects for the

oxidation of xanthine and the *N*-methylquinolinium cation, can now leave no doubt that these two classes of substrate are oxidized at the same site on the enzyme. This in turn further supports our earlier discussion (*9, 11*) of substrate binding to this enzyme in terms of a key hydrogen bond donor being present in the active site.

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