Kinetic and e.p.r. studies on the inhibition of xanthine oxidase by alloxanthine (1*H*-pyrazolo[3,4-*d*]pyrimidine-4,6-diol)

Jeffrey W. WILLIAMS* and Robert C. BRAY
*School of Molecular Sciences, University of Sussex, Falmer,
Brighton BN1 9QJ, Sussex, U.K.*

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The inhibition by alloxanthine of oxidation of xanthine by xanthine oxidase is characterized by a prolonged transient phase. Kinetic data accord with a mechanism that involves rapid formation of a reduced enzyme–alloxanthine complex that subsequently undergoes a relatively slow reversible reaction. In this scheme the slowly formed complex cannot be fully reoxidized by oxygen. From the K_i value for the dissociation of alloxanthine from the rapidly formed complex ($1.15\ \mu\text{M}$) and values of $0.37\ \text{min}^{-1}$ and $0.011\ \text{min}^{-1}$ for the forward and reverse rate constants of the slow reaction, an overall inhibition constant for alloxanthine of $35\ \text{nM}$ was calculated. A molybdenum(V) e.p.r. signal from the slowly formed reduced enzyme–alloxanthine complex is described. The rate of appearance of this new signal is consistent with this assignment. The signal (the ‘Alloxanthine signal’) was simulated with $g_1\ 2.0269$, $g_2\ 1.9593$, $g_3\ 1.9444$ and shows indications of hyperfine coupling to nitrogen. Similarities between it and the Very Rapid signal are discussed. Close structural analogies between the catalytic intermediate represented by the Very Rapid signal and the inhibitor complex represented by the Alloxanthine signal are suggested.

Allopurinol (1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol) is an effective drug for the therapy of the hyperuricaemia associated with gout or with antineoplastic or radiation therapy (Goodman & Gilman, 1975). The biochemical basis for the action of this drug is the potent inhibition of the enzyme xanthine oxidase, which catalyses the terminal step in uric acid biosynthesis. The inhibition of xanthine oxidase appears to be due to formation of a complex between the enzyme and alloxanthine (1*H*-pyrazolo[3,4-*d*]pyrimidine-4,6-diol; also known as oxipurinol), this being the product of reaction between the enzyme and allopurinol. In this complex, molybdenum in the enzyme active centre is in a reduced valence state [Mo(IV)], which is produced during the catalytic reaction (Massey *et al.*, 1970). The inhibitor complex is relatively stable in air, though it is rapidly destroyed by ferricyanide or by phenazine methosulphate (Massey *et al.*, 1970). Cha *et al.* (1975) have more recently confirmed that inhibition of xanthine oxidase by alloxanthine is markedly time-dependent. They showed that both the formation and the dissociation of the alloxanthine–enzyme complex are very slow in the presence of oxygen.

* Present address: College of Pharmacy, Ohio State University, Columbus, OH 43212, U.S.A.

Because of its pharmacological importance, we decided to investigate further the complex nature of the inhibition of xanthine oxidase by alloxanthine. By using a procedure similar to that of Williams *et al.* (1979), we now show that alloxanthine binds rapidly and reversibly to the reduced form of the enzyme and then slowly interacts to form a relatively stable inhibited complex. By the use of e.p.r. of Mo(V) in the enzyme (Bray, 1980*a,b*), we demonstrate that the slow step in the formation of the inhibitory complex is indeed the reaction of alloxanthine with a reduced state of molybdenum.

Materials and methods

General procedures

Alloxanthine and [^{14}C]allopurinol were gifts from the Wellcome Foundation, Dartford, Kent, U.K., and xanthine (sodium salt) was purchased from Sigma. Milk xanthine oxidase was prepared by the salicylate-denaturation procedure of Hart *et al.* (1970). The activity of xanthine oxidase was determined at 23.5°C , uric acid formation being monitored by A_{295} measurements on a Pye–Umicam SP.1800 recording spectrophotometer, with $90\ \mu\text{M}$

xanthine, in solutions at equilibrium with atmospheric oxygen. These and all other experiments were performed in 50 mM-sodium pyrophosphate buffer, pH 8.2. Under these conditions the change in molar absorption for conversion of xanthine into uric acid is $9.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Avis *et al.*, 1956). Reduction of xanthine oxidase with dithionite was performed anaerobically by adding a concentrated solution of the reagent from a syringe.

E.p.r. measurements were done with a Varian E9 spectrometer linked to a computer and visual-display system (Bray *et al.*, 1978), by using the techniques described by Gutteridge *et al.* (1978). Oxygenation was achieved by bubbling a slow stream of air for approx. 30 s through the samples contained in e.p.r. tubes, immediately before freezing them in liquid nitrogen for e.p.r. measurement. Stimulation of e.p.r. spectra used an iterative version (D. J. Lowe, unpublished work) of the programs of Lowe (1978). Xanthine oxidase concentrations [reported as final concentrations of functional active centres, from activity and A_{450} measurements (Bray, 1975)], in activity assays, in spectrophotometric studies and for e.p.r. measurements were respectively about 0.01, 10 and 500 μM .

Computer-sharpening of experimental e.p.r. spectra was achieved (Allen *et al.*, 1964) by further processing on the digital computer used initially to record them. After appropriate smoothing, an arbitrary fraction (e.g. 0.25) of the second derivative of the experimental spectrum was subtracted from the original spectrum, to yield the sharpened spectrum.

Tests for oxidation or degradation of alloxanthine by xanthine oxidase were performed as follows. A solution of xanthine oxidase (about 500 μM) was reduced anaerobically with 1 mM-sodium dithionite, followed by the addition of alloxanthine (2 mM). The solution was allowed to equilibrate with air for 2 days at about 20°C, then the enzyme was precipitated by addition of 2 vol. of methanol. The supernatant was chromatographed on fluorescein-treated cellulose t.l.c. plates in the solvent system of Edmondson *et al.* (1973). A similar procedure was employed with [^{14}C]allopurinol, except that, in this case, addition of dithionite to reduce the enzyme was not required. In both experiments, alloxanthine was the only compound detected at the end of the experiment.

Inhibition theory

Cha (1976) has developed a general rate equation to describe various mechanisms by which a competitive inhibitor may exhibit an inhibition which is slowly expressed. Provided that the concentration of free inhibitor is not significantly altered as a result of the formation of an enzyme-inhibitor complex, the

rate of the enzyme-catalysed reaction (v) will vary with time (t) according to the equation:

$$v = v_s + (v_0 - v_s) \exp(-k't) \quad (1)$$

In eqn. (1), v_0 is the initial observed velocity extrapolated to zero time in the presence of inhibitor, and v_s is the final steady-state rate observed in the presence of inhibitor after the entire system has come to a dynamic equilibrium. In the absence of the slowly developed inhibition, $v = v_s = v_0$. If inhibitor is omitted or does not bind to the enzyme, eqn. (1) degenerates to the simple Michaelis-Menten expression. The exact expression defining k' , an apparent first-order rate constant, will vary with the mechanism of the inhibition. Integration of eqn. (1) with respect to time gives eqn. (2), which is an expression of product concentration (P) as a function of time.

$$P = v_s t - \frac{(v_s - v_0)}{k'} [1 - \exp(-k't)] \quad (2)$$

Since P is the quantity that is measured experimentally, this is the more appropriate form of eqn. 1.

A complex enzyme such as xanthine oxidase, capable of existing in a number of different reduced forms, may in principle transform substrate into product molecules by going through alternative catalytic cycles. Thus, according to Olson *et al.* (1974b), xanthine oxidase may cycle between XO_0 and XO_{2e} , or between XO_{2e} and XO_{4e} or, somewhat less efficiently, between XO_{4e} and XO_{6e} (where XO represents the enzyme half molecule and subscripts represent numbers of electrons accepted from the fully oxidized state, XO_0). Generally, for an enzyme capable of j alternative catalytic cycles, the rate of the enzyme-catalysed reaction (v) will be given by:

$$v = \sum_0^j v_i f_i \quad (3)$$

where f_i is the proportion of the enzyme molecules that goes through a cycle whose rate is v_i .

Consider the case of two possible cycles only, referred to as 'oxidized' (i.e. cycling between XO_0 and XO_{2e}) and 'reduced' (i.e. cycling between XO_{2e} , XO_{4e} and XO_{6e}), having respectively rates $v^{\text{ox.}}$ and $v^{\text{red.}}$ and consider conditions such that a fraction, α , of the total enzyme molecules undergoes the reduced cycle. The following equations (eqns. 4–8) may be derived (cf. Cha, 1976) to describe the case when a competitive inhibitor binds rapidly to an enzyme form present in the reduced but not in the oxidized cycle, and subsequently interacts slowly to form a more tightly bound enzyme-inhibitor complex. The equations are derived by assuming $k_{\text{cat.}}$ and K_A are the same in both

enzyme cycles and that reoxidation of the final enzyme-inhibitor complex is negligibly slow:

$$v_0^{\text{ox.}} = v_s^{\text{ox.}} = \frac{V(1-\alpha)A/K_A}{1 + A/K_A} \quad (4)$$

$$v_0^{\text{red.}} = \frac{V\alpha A/K_A}{1 + A/K_A + I/K_i} \quad (5)$$

$$v_s^{\text{red.}} = \frac{V\alpha A/K_A}{1 + A/K_A + I/K_i^*} \quad (6)$$

$$\alpha = \beta_0 + (\beta_s - \beta_0)[1 - \exp(k't)] \quad (7)$$

$$k' = \frac{k_{-3}(1 + A/K_A + I/K_i^*)}{1 + A/K_A + I/K_i} \quad (8)$$

where V is $V_{\text{max.}}$, A is the concentration of substrate and K_A its Michaelis constant, I is the concentration of inhibitor and K_i ($=k_{-2}/k_{+2}$; see Scheme 1 below) the dissociation constant for any rapidly formed enzyme-inhibitor complex. In the case where there are no rapidly formed complexes, K_i would become very large and the term I/K_i in eqn. (5) would become insignificant. K_i^* [$=k_{-2}k_{-3}/k_{+2}(k_{+3} + k_{-3})$; see Scheme 1] is the dissociation constant for the more slowly formed enzyme-inhibitor complex and is comprised of K_i and the forward and reverse rate constants of the slow step. α , defined as above, varies from 0 to 1. β_0 and β_s are parameters dependent on substrate concentrations, representing limiting values of α under initial (β_0) and steady-state (β_s) conditions. In principle, β_s might also be a function of inhibitor concentration. However, if only the slowly formed enzyme-inhibitor complex remains reduced in the presence of oxygen (k_{+4} finite; see Scheme 1), the value of β_s will be determined by the rate of dissociation of this complex, which is inhibitor-independent.

The above equations are valid for two- as well as for one-substrate reactions if: (1) the second substrate is always present at saturating concentrations, or (2) the second-substrate concentration is kept constant. For the latter case, $V_{\text{max.}}$ will not represent the true value, but will instead be an apparent value for the particular conditions employed. Experiments performed with xanthine oxidase and alloxanthine fall under the second category, since oxygen, though not saturating, would not be depleted significantly.

Data analysis

A_{295} progress-curve data (obtained by reading off about 20 points each, from spectrophotometer traces from a series of experiments) were fitted by a non-linear optimization routine (Swann, 1969) to eqns. (4)–(8) given under 'Inhibition theory' above by using the STEPIT computer program written by

Dr. J. P. Chandler as modified by Dr. R. G. Duggleby (Williams *et al.*, 1980). A value of the apparent Michaelis constant for xanthine of $5\mu\text{M}$ (Tanner, 1978; cf. Olson *et al.*, 1974a) was supplied to the program, and the data were used to estimate V , K_i , β_0 , β_s and the forward and reverse first-order rate constants associated with the slow binding of alloxanthine to the enzyme. Where appropriate, apparent first-order rate data from e.p.r. or spectrophotometric experiments were fitted by non-linear regression analysis to eqn. (9).

$$P = P_\infty [1 - \exp(-kt)] \quad (9)$$

Results

Progress-curve studies

When milk xanthine oxidase is assayed with xanthine as the substrate, a steady-state rate is immediately attained and absorbance changes are linear for at least 10% of the overall reaction (Fig. 1, curve A). In the presence of alloxanthine (when

Fig. 1. Progress curves for the slow development of the inhibition of xanthine oxidase by alloxanthine. Reaction mixtures at 23.5°C contained buffer (pH 8.2), xanthine ($90\mu\text{M}$) and the following concentrations of alloxanthine (μM) in A–E respectively: 0, 39, 59, 98, 117. Reactions were initiated by the addition of enzyme (10 nM) and progress curves were obtained by monitoring A_{295} . Closed symbols (●) represent experimental points (read from spectrophotometer traces) and continuous lines are computed curves, obtained as described in the text.

starting the reaction by adding the enzyme), there is initially little effect on the reaction rate, but, as shown in Fig. 1 (curves B–E), there is a marked time-dependent decrease in the observed rate, which varies as a function of the inhibitor concentration. Preincubation of alloxanthine with enzyme in the absence of xanthine resulted in no loss of enzymic activity, and curves similar to those in Fig. 1 were observed on addition of the reducing substrate. These results support previous reports (Spector & Johns, 1968; Massey *et al.*, 1970) indicating that catalysis is necessary in order to observe the tight binding of alloxanthine to the enzyme. However, if the enzyme is first reduced anaerobically with xanthine or dithionite and then mixed with alloxanthine, there is considerable initial loss of enzymic activity. This is shown in Fig. 2, where the dithionite-reduced anaerobic preincubation mixture was added to a solution of xanthine saturated with air. The reaction rate was very low at first, but increased with time, thus indicating that alloxanthine binding is readily reversible. It also seems that only reduced enzyme forms are necessary for alloxanthine binding, rather than some intermediate produced during catalysis.

As discussed by Williams *et al.* (1979), it is necessary to determine whether the initial velocities of the progress curves in Fig. 1 vary as a function of alloxanthine concentration, in order to establish if any enzyme–inhibitor complexes are formed rapidly. To verify this point, the data for each curve of Fig. 1 were fitted separately to eqn. (2) and the reciprocals of the resulting values for v_0 were plotted against the alloxanthine concentration. As Fig. 3 shows, the

Fig. 2. Progress curves for the recovery of the activity of xanthine oxidase after reduction with dithionite (0.17 mM) and incubation with alloxanthine

Assays were initiated by addition to the reaction medium of portions of the preincubation mixture (containing enzyme and dithionite) at a 152.5-fold dilution. Final enzyme and xanthine concentrations were 10 nM and 90 μ M respectively, and alloxanthine concentrations (μ M) in A–C were respectively: 0, 5.8, 11.7. Other details are as in Fig. 1.

initial velocities (v_0) decrease with inhibitor concentration, indicating the presence of a rapidly formed enzyme–inhibitor complex with a dissociation constant of $0.8 \pm 0.3 \mu$ M.

The data are thus qualitatively consistent with the mechanism shown in Scheme 1. Table 1 lists the values of the kinetic constants associated with this

Fig. 3. Dixon plot of the effect of alloxanthine on the initial velocities (v_0) of reactions initiated by the addition of xanthine oxidase

The initial velocities were determined by computer fitting to eqn. (2) of progress-curve data from experiments such as those in Fig. 1.

Scheme 1. Kinetic scheme for interaction of alloxanthine with xanthine oxidase during turnover of xanthine in the presence of oxygen

See the text for details. Abbreviation used: Xan., xanthine.

Table 1. *Kinetic constants of xanthine oxidase as determined by progress-curve analysis*

Progress-curve data from a series of experiments such as those depicted in Figs. 1 and 2 were analysed by fitting to the mechanism of Scheme 1 by using the STEPIT program. The concentrations of xanthine and xanthine oxidase were 90 μM and 10 nM respectively. The error values are standard errors from overall fits, except for k_{-3} , which is reported as the S.D. of three separate determinations of this value. See the text for further details and for definition of β_0 and β_s . E_t is the total enzyme concentration (functional active centres).

Parameter	Value	Error
$V/(E_t)$ (min^{-1})	777*	10
$K_1^\dagger$ (μM)	1.15	0.04
k_{+3} (min^{-1})	0.37	0.01
k_{-3} (min^{-1})	0.011	0.002
β_0	0.53	0.01
β_s	0.95	0.02

* This parameter corresponds to the catalytic-centre activity. The value is to be compared with one of 720 min^{-1} quoted by Bray (1975).

$^\dagger K_1$ represents the ratio k_{-2}/k_{+2} .

mechanism, which were determined by computer analysis of the progress-curve data according to the procedure outlined under 'Inhibition theory' and 'Data analysis'. The value of k_{-3} was not well determined from the data in Fig. 1. However, a more precise estimate of this constant (consistent with Fig. 1) was obtained from the data in Fig. 2, by holding constant V , K_1 and k_{+3} at the values given in Table 1. This has been shown to be a valid procedure for the determination of the corresponding rate constant for dihydrofolate reductase (Williams *et al.*, 1979).

E.p.r. studies

It would be of particular interest if a specific Mo(V) e.p.r. signal could be associated with the slowly formed alloxanthine-enzyme complex, since such a signal might be useful in identifying the exact chemical nature of the complex. A preliminary description of a signal apparently related to the complex has in fact been presented (Tanner, 1978).

Swann & Bray (1972) found in the early stages of allopurinol turnover to alloxanthine by xanthine oxidase, that, not unexpectedly, a Rapid Mo(V) signal may be observed. Also, as reported previously (Massey *et al.*, 1970; Swann & Bray, 1972) and confirmed in the present work, incubation of the enzyme with excess xanthine in the presence of alloxanthine results in the appearance of a flavin-radical signal, sometimes accompanied by varying amounts of the Slow Mo(V) signal from the desulpho enzyme. In contrast, however, we found

Fig. 4. *E.p.r. spectrum of alloxanthine-inhibited xanthine oxidase showing the 'Alloxanthine signal'* (b) shows the complete experimental spectrum, (a) is a computer simulation of this and (c) shows the high-field part of (b), replotted on a 2-fold expanded horizontal scale. (d) Shows spectrum (c) replotted after having been submitted to computer line-sharpening (see the Materials and methods section). For (b), (c) and (d), the spectrum was recorded at 9.377 GHz and about 150 K with 10 mW microwave power and 0.25 mT modulation amplitude. The sample in 50 mM-sodium pyrophosphate buffer, pH 8.2, was first reduced with xanthine, then treated with alloxanthine and finally aerated briefly before being frozen in liquid N_2 (see the Materials and methods section). The simulation (a) was based on the following parameters (hyperfine splittings and half-linewidths, Δ , in mT): $g_{1,2,3}$ 2.0269, 1.9593, 1.9444; $A(^{14}\text{N})_{1,2,3}$ 0.33, 0.36, 0.39; $\Delta_{1,2,3}$ 0.19, 0.16, 0.19. The arrows correspond, for (a) and (b), to $g = 2.0037$.

that if such a mixture was briefly equilibrated with air before freezing (see the Materials and methods section), then the flavin and Slow signals disappeared and there appeared a novel molybdenum(V) signal (Fig. 4b; cf. Tanner, 1978). This we ascribe to an oxidation product of the initially formed, e.p.r.-undetectable, alloxanthine-Mo(IV) enzyme complex.

The new signal (Fig. 4b), though exhibiting no resolved hyperfine structure, has a larger apparent linewidth than have other Mo(V) e.p.r. spectra from enzymes (Bray, 1980a). Change of the solvent from $^1\text{H}_2\text{O}$ to $^2\text{H}_2\text{O}$ resulted in no detectable change in form, indicating the absence of coupling to exchangeable protons. However, when the spectrum of Fig. 4(b) (or similar spectra from other comparable samples) was submitted to a computer line-sharpen-

ing procedure (see the Materials and methods section for details), the g_2 feature showed indications of becoming resolved into three lines (compare Figs. 4c and 4d), suggesting coupling of molybdenum to a single nitrogen nucleus. Such coupling has been included in the simulation of the spectrum shown in Fig. 4(a), which indicates g_1 2.0269, g_2 1.9593, g_3 1.9444 and g_{av} 1.9769.

Intensity of the signal was relatively low, double integration indicating, in one experiment, involvement of about 2% of the molybdenum of the functional enzyme. In other experiments (not illustrated) in which the reaction period for reduced enzyme with alloxanthine was varied, it was shown that the new e.p.r. signal (which we shall call the 'Alloxanthine signal') developed rather slowly. From three such experiments at different inhibitor concentrations it was possible to calculate, from the vertical intercept of a double-reciprocal plot (Fig. 5), a first-order rate constant of $0.38 \pm 0.08 \text{ min}^{-1}$ for formation of the Alloxanthine signal at saturating concentrations of alloxanthine.

Spectrophotometric studies

In confirmation of the results of Massey *et al.* (1970), we observed a difference spectrum between untreated enzyme and enzyme first reduced anaerobically with xanthine, then treated with alloxanthine

and finally reoxidized with air. When such difference spectra were obtained from samples incubated for various periods with alloxanthine at 21°C , it was noted that the intensity of the difference spectrum (measured as the absorbance difference between the peak at 380nm and the trough at 450nm) slowly increased as a function of reaction time with alloxanthine. An apparent first-order rate constant of $0.47 \pm 0.10 \text{ min}^{-1}$ was determined from the data.

Discussion

Figs. 1 and 2 clearly indicate that alloxanthine may be classified as a slow-binding inhibitor that is not necessarily tight-binding or stoichiometric (Williams & Morrison, 1979). Indeed, the concentration of alloxanthine in the progress-curve experiments was always much higher than the enzyme concentration. It thus appears that it is the very slow rate of dissociation of alloxanthine from the enzyme (k_{-3}) that accounts for the stoichiometric behaviour reported previously (Spector & Johns, 1968; Massey *et al.*, 1970). However, the value of the overall dissociation constant calculated from our data (K_i^*) is certainly not in the range of a truly tight-binding inhibitor (Williams & Morrison, 1979). Cha *et al.* (1975) reported observations similar to the present ones, although they made no attempt to analyse their data quantitatively.

Our results further support the thesis that the enzyme must be in the reduced state before it can be inhibited by alloxanthine (Spector & Johns, 1968; Massey *et al.*, 1970). This point, together with the evidence for a rapidly formed enzyme-inhibitor complex (Fig. 3), suggested the complex mechanism for the alloxanthine inhibition that is outlined in Scheme 1. The mechanism involves the rapid reaction of the inhibitor with a reduced enzyme species to form an EI complex that then undergoes a slow, reversible reaction to form an EI* complex. According to Scheme 1, the EI* complex cannot be completely reoxidized by oxygen. Analysis of the progress-curve data according to this mechanism yielded estimates of the parameters in Scheme 1 (Table 1) with low standard errors. A much poorer fit to the data was obtained if alloxanthine was considered to react rapidly during the oxidized as well as during the reduced catalytic cycles. Thus the data appear both qualitatively and quantitatively in agreement with the mechanism of Scheme 1.

From the values in Table 1 it is possible to calculate the overall inhibition constant (K_i^*) for the combination of alloxanthine with the enzyme according to the relationship $K_i^* = K_i k_{-3} / (k_{+3} + k_{-3}) = 34.7 \text{ nM}$. This value is an order of magnitude higher than the value determined by Spector & Johns (1970), who used classical techniques, which are, however, not expected to yield reliable results

Fig. 5. Determination of the first-order rate constant for the formation of the e.p.r.-detectable alloxanthine-enzyme complex

The reciprocals of apparent first-order rate constants derived from measurements of intensity of the Alloxanthine signal are plotted against the reciprocal of the alloxanthine concentration. The experiments were performed at 21°C and the spectra recorded as for Fig. 4, the incubation time of alloxanthine with reduced enzyme being varied.

for slowly expressed inhibition (Williams & Morrison, 1979).

Massey *et al.* (1970) indicated that, in the presence of oxygen, the reduced enzyme–alloxanthine complex became re-activated, with a rate constant of 0.002 min^{-1} . This value is five times lower than k_{-3} , the rate constant for reversal of the slow step of Scheme 1. Since these workers did not specify inhibitor or substrate concentrations, it is, however, difficult to draw detailed conclusions.

The values of the parameters β_0 and β_s (as defined above under 'Inhibition theory') in Table 1 provide information respectively about the average oxidation state of the enzyme immediately on mixing with the substrate and inhibitor, and in the final steady state. The value of β_0 (0.53 ± 0.01) is in agreement with the well-known partial reduction of the enzyme during uninhibited steady-state turnover in air equilibrated buffer (Olson *et al.*, 1974b). β_s (0.95 ± 0.02), on the other hand, reflects the large extent to which the formation of the enzyme–inhibitor complex traps the enzyme in reduced forms in the steady state and had not been measured previously.

The absorption-difference spectrum, having a peak at about 380 nm, between native enzyme and enzyme treated in the reduced state with alloxanthine then re-oxidized briefly with air, has been attributed to the alloxanthine–enzyme complex (Massey *et al.*, 1970). Indeed, the apparent rate constant with which the absorption peak appears (0.47 min^{-1}) is almost identical with the value of k_{+3} , thus supporting the previous report and further validating the kinetic analysis above. Unfortunately this spectrum cannot as yet be directly attributed to a particular chromophore. It was therefore particularly noteworthy that comparable treatment of the enzyme gave rise to a specific Mo(V) e.p.r. signal, the Alloxanthine signal. Existence of such a signal constitutes the first direct proof that the inhibitor is closely associated with the metal in the inhibited complex. The rate of formation of the Alloxanthine signal ($k = 0.38 \pm 0.08 \text{ min}^{-1}$) is also consistent with the value of k_{+3} determined from activity measurements. Thus it is the reaction of alloxanthine with molybdenum, or with a group very near to molybdenum, which is responsible for the unusual kinetic characteristics of the inhibition. Failure to observe the Alloxanthine signal in mixtures of the enzyme with xanthine and alloxanthine from which oxygen has become depleted, and conversely, appearance of the signal on aeration [or, according to Tanner (1978), on treatment with ferricyanide] necessitate postulation of one-electron oxidation and reduction reactions controlled by k_{+5} and k_{-5} (Scheme 1). Indications are that the apparent value of k_{+5} in the presence of oxygen is relatively high.

Finally we consider the parameters of the Alloxanthine signal and the bearing that these have on the structure of the inhibited complex. The lack of hyperfine splitting due to coupling to exchangeable protons and the high value of g_1 (2.0269) at once suggests an analogy with the Very Rapid signal. The latter, with g_1 2.0252 (Bray, 1980a), was up until now the only Mo(V) signal from xanthine oxidase to show a principal g -value greater than the free-electron value. The linewidth of the signal, together with the results from computer sharpening, suggest hyperfine coupling of molybdenum to nitrogen. The simulation shown in Fig. 4(a) was performed, starting from the linewidths of the Very Rapid signal and provides some further support for such coupling. To arrive at a satisfactory simulation, it was necessary to assume almost isotropic coupling to a nitrogen atom with $A(^{14}\text{N})_{\text{av}}$ 0.36 mT.

Confirmation that the Alloxanthine signal-giving species is to be thought of as an analogue of the species giving Very Rapid rather than of that giving Inhibited, might in principle be achieved by substitution of the enzyme with ^{33}S (Malthouse & Bray, 1980). Both Very Rapid and a further new analogue of this signal (particularly like the Alloxanthine signal) obtained with the inhibitor and substrate 2-amino-4-hydroxy-6-formylpteridine show strong coupling of ^{33}S to Mo(V). On the other hand, Inhibited shows only very weak coupling (J. P. G. Malthouse, J. W. Williams and R. C. Bray, unpublished work). Unfortunately, the large linewidth of the Alloxanthine signal has so far prevented us extending ^{33}S studies to this species.

If, nevertheless, we accept an analogy with Very Rapid, a number of points follow. Very Rapid is believed (Bray, 1980b) to represent an early and transient intermediate in the turnover of substrates such as xanthine. The structure of the signal-giving species has been suggested, on the basis of work with ^{17}O (Gutteridge & Bray, 1980), to be as shown in Scheme 2(a). This species would arise via nucleophilic attack on the substrate by a terminal oxygen ligand of molybdenum, leading to elimination of a hydride equivalent from the substrate molecule. The resulting carbonium ion would be stabilized by bonding to the oxygen, as indicated. The similarity of the Alloxanthine signal suggests that alloxanthine is acting like a substrate, but becomes trapped on the enzyme as an unreactive species, having a structure analogous to that in Scheme 2(a). We confirmed the previous finding by Massey *et al.* (1970) that alloxanthine may be recovered unchanged after treatment with xanthine oxidase. This, and failure of the inhibitor to reduce the enzyme, clearly mean that hydride elimination from alloxanthine does not have to be considered. However, as shown in Scheme 2(b), an alloxanthine tautomer having no proton in the 2-position might bind to the pre-reduced enzyme

Scheme 2. Possible structures of Mo(V) e.p.r. signal-giving species

(a) Represents the Very Rapid signal (as suggested by Gutteridge & Bray, 1980) with the xanthine residue linked covalently to the enzyme through its 8-position and (b) represents the Alloxanthine signal in which the appropriate tautomer of the inhibitor molecule is correspondingly placed in the active centre. Both substrate and inhibitor would of course be positioned by interaction with other groups in the active centre and might also be co-ordinated to molybdenum.

in a manner analogous to that in the Very Rapid species. Coupling of the N-2 nitrogen atom to Mo(V) could then explain the linewidth of the Alloxanthine signal and would be analogous to C-8 coupling in Very Rapid (Tanner *et al.*, 1978). The slow process represented by k_{+3} might involve a rearrangement of the reduced active centre or slow tautomerization of the enzyme-bound alloxanthine to the form indicated. Failure of the Alloxanthine-signal-giving species to break down in the manner proposed (Gutteridge & Bray, 1980) for Very Rapid, that is via attack by a hydroxyl ion on molybdenum and fission of the Mo—O bond, would be related both to the high stability of the uric acid anion in comparison with that of the corresponding hypothetical alloxanthine N-oxide and to the low stability of the xanthine carbonium ion in comparison with alloxanthine.

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References

- Allen, L. C., Gladney, H. M. & Glarum, S. H. (1964) *J. Chem. Phys.* **40**, 3135–3141
- Avis, P. G., Bergel, F. & Bray, R. C. (1956) *J. Chem. Soc.* 1219–1226
- Bray, R. C. (1975) *Enzymes 3rd Ed.* **12**, 299–419
- Bray, R. C. (1980a) in *Biological Magnetic Resonance*, vol. 2 (Berliner, L. J. & Reuben, J., eds.), pp. 45–83, Plenum Press, New York
- Bray, R. C. (1980b) *Adv. Enzymol.* **51**, 107–165
- Bray, R. C., Barber, M. J. & Lowe, D. J. (1978) *Biochem. J.* **171**, 653–658
- Cha, S. (1976) *Biochem. Pharmacol.* **25**, 2695–2702
- Cha, S., Agarwal, R. P. & Parks, R. E. (1975) *Biochem. Pharmacol.* **24**, 2187–2197
- Edmondson, D., Ballou, D., Van Heuvelen, A., Palmer, G. & Massey, V. (1973) *J. Biol. Chem.* **248**, 6135–6144
- Goodman, L. S. & Gilman, A. (1975) *The Pharmacological Basis of Therapeutics* 5th edn., pp. 352–355, Macmillan Publishing Co., New York
- Gutteridge, S. & Bray, R. C. (1980) *Biochem. J.* **189**, 615–623
- Gutteridge, S., Tanner, S. J. & Bray, R. C. (1978) *Biochem. J.* **175**, 869–878
- Hart, L. I., McGartoll, M. A., Chapman, H. R. & Bray, R. C. (1970) *Biochem. J.* **116**, 851–864
- Lowe, D. J. (1978) *Biochem. J.* **171**, 649–651
- Malthouse, J. P. G. & Bray, R. C. (1980) *Biochem. J.* **191**, 265–267
- Massey, V., Komai, H., Palmer, G. & Elion, G. B. (1970) *J. Biol. Chem.* **245**, 2837–2844
- Olson, J. S., Ballou, D. P., Palmer, G. & Massey, V. (1974a) *J. Biol. Chem.* **249**, 4350–4362
- Olson, J. S., Ballou, D. P., Palmer, G. & Massey, V. (1974b) *J. Biol. Chem.* **249**, 4363–4382
- Spector, T. & Johns, D. G. (1968) *Biochem. Biophys. Res. Commun.* **32**, 1039–1044
- Spector, T. & Johns, D. G. (1970) *J. Biol. Chem.* **245**, 5079–5085
- Swann, J. C. & Bray, R. C. (1972) *Eur. J. Biochem.* **26**, 407–415
- Swann, W. H. (1969) *FEBS Lett. Suppl.* **2**, S39–S55
- Tanner, S. J. (1978) D.Phil. Thesis, University of Sussex
- Tanner, S. J., Bray, R. C. & Bergmann, F. (1978) *Biochem. Soc. Trans.* **6**, 1328–1330
- Williams, J. W. & Morrison, J. F. (1979) *Methods Enzymol.* **63**, 437–467
- Williams, J. W., Morrison, J. F. & Duggleby, R. G. (1979) *Biochemistry* **18**, 2567–2573
- Williams, J. W., Duggleby, R. G., Cutler, R. & Morrison, J. F. (1980) *Biochem. Pharmacol.* **29**, 589–595