The structure of the inhibitory complex of alloxanthine (1*H*-pyrazolo[3,4-*d*]pyrimidine-4,6-diol) with the molybdenum centre of xanthine oxidase from electron-paramagnetic-resonance spectroscopy

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Studies were carried out on the inhibitory complex of alloxanthine (1*H*-pyrazolo[3,4-*d*]pyrimidine-4,5-diol) with xanthine oxidase, in extension of the work of Williams & Bray [*Biochem. J.* (1981) **195**, 753–760]. By suitable regulation of the reaction conditions, up to 10% of the functional enzyme could be converted into the complex in the Mo(V) oxidation state. The e.p.r. spectrum of the complex was investigated in detail with the help of computer simulation and substitution with stable isotopes. Close structural analogy of the signal-giving species to that of the Very Rapid intermediate in enzyme turnover is shown by *g*-values (2.0279, 1.9593 and 1.9442) and by coupling to ³³S in the cyanide-labile site of the enzyme [*A*(³³S) 0.30, 3.10 and 0.70 mT]. However, whereas in the Very Rapid signal there is strong coupling to ¹⁷O [Gutteridge & Bray, *Biochem. J.* (1980) **189**, 615–623], instead, in the Alloxanthine signal there is strong coupling to a single nitrogen atom [*A*(¹⁴N) 0.35, 0.35, 0.32 mT]. This is presumed to originate from the 2-position of the heterocyclic ring system. From this work and from earlier kinetic studies it is concluded that alloxanthine, after being bound reversibly at the active centre, reacts slowly with it, in a specific manner, distinct from that in the normal catalytic reaction with substrates. This reaction involves elimination of an oxygen ligand of molybdenum and co-ordination, in this site, of alloxanthine via the N-2 nitrogen atom, to give a complex that is structurally but not chemically closely analogous to that of the Very Rapid species.

Allopurinol (1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol), which is used clinically in the treatment of hyperuricaemia (see, e.g., Wade, 1977), is converted *in vivo* by the action of xanthine oxidase into alloxanthine (1*H*-pyrazolo[3,4-*d*]pyrimidine-4,6-diol). The action of the drug is due to the latter compound forming a very tight complex with the reduced form of the molybdenum centre of xanthine oxidase and thus effectively inhibiting the action of the enzyme (Massey *et al.*, 1970).

Considerable progress has been made in recent years towards elucidation of the structures of molybdenum centres in enzymes, by e.p.r. spectroscopy of the centres in the Mo(V) oxidation state (see, e.g. Bray, 1980*a,b*; Bray & Gutteridge, 1982; George & Bray, 1983*a,b*; Bray *et al.*, 1983). Though the alloxanthine complex of xanthine oxidase, as

initially prepared, contains molybdenum, at least predominantly, in the Mo(IV) oxidation state (Massey *et al.*, 1970), nevertheless it is possible (Tanner, 1978; Williams & Bray, 1981), by suitable oxidation, to convert some of the metal into the Mo(V) state. The complex then gives rise to a specific e.p.r. signal, designated the Alloxanthine signal. We here present additional studies of this signal, permitting us to deduce the structure of the inhibitory complex and to propose a mechanism for the inhibitory reaction.

Materials and methods

Materials

Alloxanthine was a gift from the Wellcome Foundation (Dartford, Kent, U.K.), water enriched with ¹⁷O was obtained from Monsanto Research Corp., Mound Facility, Miamisburg, OH, U.S.A., and ³³S-enriched elemental sulphur from Oak Ridge National Laboratory, Oak Ridge,

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TN, U.S.A. The latter was converted into S^{2-} as described by Malthouse & Bray (1980). Mediator redox dyes were as follows: *p*-benzoquinone (laboratory-reagent grade), Indigo Carmine and phenosafranine (BDH Chemicals, Poole, Dorset, U.K.), galloxyaniline (Hopkin and Williams, Dagenham, Essex, U.K.), 1,2-naphthoquinone (Cambrian Chemicals, Croydon, Surrey, U.K.) and 2,5-dihydroxy-*p*-benzoquinone (Eastman Kodak, Rochester, NY, U.S.A.).

Xanthine oxidase

Xanthine oxidase was prepared and concentrations of the functional enzyme were determined as described in previous publications (Hart *et al.*, 1970; Bray, 1975, 1982). Xanthine oxidase enriched in the cyanide-labile site with ^{33}S (59% enrichment) was prepared by a minor modification (A. Ventom, unpublished work) of the method of Malthouse & Bray (1980) by reconstitution of the desulpho enzyme with ^{33}S sulphide to yield enzyme that was 50% functional. Xanthine oxidase enriched with ^{17}O (54% enrichment) in the site or sites coupled to molybdenum was prepared (Gutteridge & Bray, 1980) by dissolving the freeze-dried enzyme in ^{17}O -containing water.

Generation of the Alloxanthine Mo(V) e.p.r. signal

Alloxanthine (final concentration 10 mM) was added to xanthine oxidase {final concentration up to 0.8 mM in 50 mM-Bicine [*NN*-bis-(2-hydroxyethyl)glycine]/NaOH buffer, pH 8.2–8.5}. The mixture was transferred to a potentiometric titration cell at 20–25°C, as described by Cammack *et al.* (1976), equipped with platinum and calomel electrodes and flushed with purified N_2 . The potential between the electrodes was measured with a millivoltmeter (1 G Ω input impedance; redox potentials are expressed relative to the hydrogen electrode). The sample was first reduced by addition of concentrated $\text{Na}_2\text{S}_2\text{O}_4$ solution (final concentration approx. 5 mM; potential about –450 mV). After 15 min, mediator dyes were added (about 0.1 mM of each of the six dyes named above), and the redox potential was then cautiously raised to between +100 and +300 mV by the addition of $\text{K}_3[\text{Fe}(\text{CN})_6]$ solution. The potential gradually decreased as the reaction proceeded, and was maintained within this range for 90 min by making small further additions of $\text{K}_3[\text{Fe}(\text{CN})_6]$ at approx. 5 min intervals. At the end of this time (and with the potential at the upper end of the range), a sample was withdrawn into an e.p.r. tube and frozen in liquid N_2 .

The above procedure was used without modification for ^{33}S -enriched enzyme. For the ^{17}O -enriched enzyme the pH value of the freeze-dried enzyme in ^{17}O -enriched water was first adjusted

with Na_2CO_3 ; $\text{Na}_2\text{S}_2\text{O}_4$ was employed as the solid rather than as a solution, and $\text{K}_3[\text{Fe}(\text{CN})_6]$ was made up in ^{17}O -enriched water.

E.p.r. measurements

E.p.r. spectra were recorded on a Varian E9 spectrometer equipped with a dual-sample cavity and linked to a DEC PDP 11/10 computer and visual display system (Bray *et al.*, 1978). For further manipulations and for simulation, e.p.r. spectra were transferred to the University of Sussex DEC VAX 11/780 computer via a link. Simulation routines (George, 1983; G. N. George, unpublished work) allowed for non-coincident axes of *g* and *A* and included second-order terms. Recording conditions for e.p.r. spectra were generally 120 K, microwave power 10 mW, modulation amplitude 0.16 mT, and microwave frequency about 9.3 GHz.

Results

Conversion of the inhibitory complex with alloxanthine into the Mo(V) oxidation state

The Alloxanthine signal, as obtained by Williams & Bray (1981), was very weak, corresponding to only about 2% conversion of molybdenum in active xanthine oxidase into the signal-giving species. For more detailed e.p.r. studies it was necessary to achieve maximum possible conversion of the enzyme into this state, and thus to maximize signal intensity.

The basic procedure for obtaining the signal from alloxanthine involves treatment of the enzyme with this inhibitor in the presence of a reducing agent, which converts molybdenum into Mo(IV) and thus permits formation of the complex. We used dithionite as the reducing agent. Controlled re-oxidation of the complex so formed leads to complete oxidation of the flavin and iron-sulphur centres and to conversion of some Mo(IV) into the Mo(V) signal-giving species. Over-oxidation results (Massey *et al.*, 1970) in re-conversion into the Mo(VI) state, with destruction of the inhibitory complex, the enzyme being re-activated in the process and the alloxanthine being then recoverable unchanged from the reaction mixture.

Williams & Bray (1981) used air for oxidation of the complex. However, we were able to obtain stronger Alloxanthine signals by reverting to a procedure more like that originally described by Tanner (1978), in which ferricyanide was employed, in the presence of mediator dyes. We found that, with careful adherence to the procedure as described in the Materials and methods section, conversion into the signal-giving species of about 10% of the active enzyme could be achieved.

Fig. 1. E.p.r. spectra of the Alloxanthine signal in the normal enzyme and after enrichment with ^{17}O or with ^{33}S

The spectra were generated, recorded and simulated (by using the parameters listed in Table 1) as described in the text. (a) shows the spectrum from normal enzyme, and (c) is the computer simulation of this. (b) is the spectrum from enzyme enriched with ^{17}O (54% enrichment). (d) shows the spectrum of enzyme enriched (59%) with ^{33}S , and (e) is the

E.p.r. parameters of the Alloxanthine signal and isotopic-substitution studies

The Alloxanthine signal is shown in Fig. 1(a). Williams & Bray (1981) suggested that the unusually large linewidth is due to unresolved hyperfine structure from nitrogen, and obtained some support for this from computer line-sharpening studies. More definitive information, confirming coupling to a single nitrogen atom, is provided by the present data, which employed spectra recorded with much improved signal-to-noise ratios. We used second-derivative presentation of spectra (i.e. third derivative of absorption spectra) and smoothing to show up unresolved hyperfine structure. Figs. 2(a) and 2(c) show the second derivative of the g_2 and g_3 region of Fig. 1(a). Three major upward features in the g_2 region stand out above the noise level in Fig. 2(a) and, after smoothing, three lines are also clearly visible in the g_3 region (Fig. 2c). Figs. 1(c) and 2(d) show the corresponding simulations, based on the parameters of Table 1, and assuming a single coupled nitrogen atom. The fit to the experimental spectra is excellent, both in the spectra as recorded and in second-derivative form.

Xanthine oxidase that has been equilibrated with ^{17}O -enriched water and that is subsequently

simulation of this. The stick diagrams here and in Fig. 2 show the g -values and the hyperfine coupling to nitrogen. The arrow corresponds to $g = 2.0037$.

Table 1. E.p.r. parameters of the Alloxanthine and the xanthine Very Rapid Mo(V) e.p.r. signals

Parameters of the Alloxanthine signal are the ones used in the simulations, carried out as described in the Materials and methods section, and illustrated in Figs. 1(c), 1(e), 2(d) and 3(f). Data on the xanthine Very Rapid signal from the literature are shown for comparison. Hyperfine splittings, A , and half-linewidths, Δ , are in mT. g -values are believed to be accurate to ± 0.0003 . Angles $X-O-X'$, $Y-O-Y'$ and $Z-O-Z'$ are angles of non-coincidence, where X , Y and Z refer to the axes (3, 2, 1) in which g is diagonal and X' , Y' and Z' refer to the axes in which $A(^{33}\text{S})$ is diagonal.

Signal	Parameter	1 (Z-O-Z')	2 (Y-O-Y')	3 (X-O-X')	Average	Reference
Alloxanthine	g	2.0279	1.9593	1.9442	1.9771	Present work
	$A(^{14}\text{N})$	0.35	0.35	0.32	0.34	Present work
	$A(^{33}\text{S})$	0.30	3.10	0.70	1.37	Present work
	Angles (^{33}S)	10°	51°	50°	—	Present work
	$A(^{17}\text{O})$	(Not observed)				Present work
	Δ^*	0.19	0.17	0.20	0.19	Present work
	Δ	0.19	0.17	0.20	0.19	Present work
Very Rapid (xanthine)	g	2.0252	1.9550	1.9494	1.9765	Tanner <i>et al.</i> (1978)
	$A(^{14}\text{N})$	(Not observed)				
	$A(^{33}\text{S})$	0.30	2.80	0.70	1.27	Malthouse <i>et al.</i> (1981)
	Angles (^{33}S)	10°	41°	40°	—	Malthouse <i>et al.</i> (1981)
	$A(^{17}\text{O})$	1.34	1.40	1.36	1.37	Gutteridge & Bray (1980)
	Δ	0.19	0.16	0.19	0.18	Tanner <i>et al.</i> (1978)

* For the simulation of Fig. 2(d), Lorentzian lineshape was used, in place of the normal Gaussian shape, and the widths were multiplied by the factor 1.7.

Fig. 2. Second derivative of e.p.r. spectra of the Alloxanthine signal

Second-derivative spectra (third derivation of absorption spectra) were generated from spectra as presented in Fig. 1, and reveal unresolved hyperfine coupling to nitrogen and the absence of unresolved hyperfine coupling to oxygen. (a), (b) and (d) correspond to the g_2 and g_3 region only respectively of the spectra of the normal enzyme, the ^{17}O -enriched enzyme and the computer simulation [as from Figs. 1(a), 1(b) and 1(c) respectively]. (c) is a smoothed version of (a) and was obtained by the use of Fourier filtering techniques.

reduced to the Mo(V) state shows clear coupling of the metal to this isotope in all the main signal-giving species (Bray & Gutteridge, 1982). We carried out the exchange procedure with ^{17}O , as described by these workers, then developed the Alloxanthine signal. As shown in Figs. 1(b) and 2(b), no evidence whatever was obtained for coupling of ^{17}O to molybdenum, the spectra, both as recorded and in second-derivative form, being indistinguishable from those obtained in ordinary water (Figs. 1a and 2a). Simulations indicated that a ^{17}O -splitting of about 0.02 mT would have been detectable, so that any coupling to this isotope must be less than this value. That our failure to observe such coupling was not due to failure of the enzyme (at least in the desulpho form), in this particular experiment, to exchange with the solvent is shown by the finding (not illustrated)

that, on incubation after further addition of dithionite to the solution used to record Fig. 1(b), the Slow signal developed and showed the expected ^{17}O -hyperfine pattern (Bray & Gutteridge, 1982).

Finally, we developed the Alloxanthine signal for a sample of enzyme that had been enriched (Malthouse *et al.*, 1981), in the cyanide-labile site, with ^{33}S . Fig. 1(d) shows the striking effect of this isotope on appearance of the signal. Fig. 1(e) is a simulation based on the parameters of Table 1 and allowing for non-coincident axes of g and A . [The simulation of Fig. 1(e) corresponds to the enrichment of ^{33}S actually used, 59%, i.e. the simulated enriched and unenriched spectra have been added together in appropriate proportions.] Fig. 3(e) shows, in the g_1 region only, the difference spectrum corresponding to 100% enrichment with

Fig. 3. Comparison of hyperfine coupling to ^{33}S in the Alloxanthine and the Very Rapid signals

Only the g_1 features of the spectra are shown. (d)–(f) show the Alloxanthine signal (samples as in Fig. 1), and (a)–(c) show the xanthine Very Rapid signal (data replotted from Malthouse *et al.*, 1981). (a) and (d) show spectra from the normal enzyme, (b) and (e) spectra from ^{33}S -enriched enzyme (difference spectra corresponding to 100% enrichment), and (c) and (f) are simulations corresponding respectively to (b) and (e).

^{33}S . Fig. 3(f) is the corresponding simulation, and Fig. 3(d) is the experimental unenriched spectrum (cf. Fig. 1a). For comparison, corresponding spectra for the g_1 feature of the xanthine Very Rapid signal are shown in Figs. 3(a)–3(c).

Discussion

The present data confirm and extend the suggestion by Williams & Bray (1981) that the Alloxanthine signal is to be regarded as an analogue of the Very Rapid signal. The latter signal, as obtained with xanthine, represents (Bray, 1980a) a transient intermediate in enzyme turnover, with a life-time (at 20°C) of a few tens of milliseconds. It has more recently come to light that the Very Rapid signal is not unique to xanthine but that this is one of a large family of Very Rapid signals, obtainable with a considerable range of substrates. These include an aldehyde-like substrate, and some of the signals are considerably longer-lived than that from xanthine (G. N. George, F. F. Morpeth & R. C. Bray, unpublished work). Though the g_{av} of the xanthine Very Rapid

signal (Bray, 1980b) is similar to that of other Mo(V) signals from active xanthine oxidase, nevertheless other parameters of the signal are unique in several ways. Notable among these are the large anisotropy of the g -values (large difference between g_1 and g_3) and the high value of g_1 , which is considerably above the free-electron value. A further unique feature concerns coupling to ^{33}S in enzyme substituted with this isotope in the cyanide-labile site, identified (Bordas *et al.*, 1980; Cramer *et al.*, 1981), in the oxidized state of the enzyme, as a Mo=S grouping. This coupling is much larger (Malthouse *et al.*, 1981) in the Very Rapid signal than it is in other signals from active xanthine oxidase. The coupling to ^{33}S is also highly anisotropic in the Very Rapid signal, with the axes of g and A substantially rotated relative to one another.

As shown in Table 1, in which the parameters of the Alloxanthine signal are compared with those of the xanthine Very Rapid signal, the Alloxanthine signal mimics the Very Rapid signal in all of these features. The data relating to ^{33}S are brought out in further detail in Fig. 3, where the g_1 features of the two signals are compared. For both signals the non-coincidence of the axes results in the four hyperfine lines not being centred on the g -value and in their being bunched together towards the high-field end of the spectrum. The close similarity of the angular relationships in the two spectra is quantified in terms of the similarity of the corresponding angles, as listed in Table 1. Indeed this Table makes abundantly clear the basic similarity of the Alloxanthine and Very Rapid signals with regard to g -values, ^{33}S couplings and linewidths.

Notwithstanding the above similarities, the parameters of the Alloxanthine and Very Rapid signals differ (Table 1) in the important respects that the former shows hyperfine coupling to nitrogen and none to oxygen, whereas in the latter this situation is reversed. Since similar e.p.r. parameters (at the level of similarity being considered here) implies similar structures of the signal-giving species, we may at once consider the possibility that in the Alloxanthine signal-giving species a nitrogen atom has taken the place of an oxygen atom that is present in the Very Rapid signal-giving species. In support of this, both of these sets of hyperfine coupling appear (Table 1) to be essentially isotropic. The relative magnitudes of the couplings further support this suggestion. According to the data of Goodman & Raynor (1970), for equal electron delocalization on to ligands, hyperfine couplings to oxygen should be about 3.0 times greater than those for nitrogen. In reasonable agreement, in Table 1, A_{av} for coupling of oxygen in the Very Rapid signal is 3.8 times that to nitrogen in the Alloxanthine signal. We there-

fore conclude that the structure of the Alloxanthine signal-giving species is closely similar to that of the Very Rapid signal-giving species, but with a nitrogen atom in the former replacing an oxygen atom in the latter.

The structure of the Very Rapid-signal-giving species (Tanner *et al.*, 1978; Gutteridge & Bray, 1980; Bray & Gutteridge, 1982) is shown in Fig. 4(a). The coupled oxygen atom is taken to be the one present as Mo=O in the oxidized enzyme (Bordas *et al.*, 1980; Cramer *et al.*, 1981) and transferred to the substrate in turnover, since it exchanges rapidly with solvent if the enzyme is reduced with a substrate and re-oxidized, before generation of the Very Rapid signal (Gutteridge & Bray, 1980). The C-8 carbon atom of the xanthine molecule shows weak ^{13}C -coupling to the metal (Tanner *et al.*, 1978). The coupled sulphur is taken to be Mo=S rather than Mo-S-, since the strong ^{33}S -coupling is anisotropic (Malthouse *et al.*, 1981), as is expected for a ligand of this type.

We have to consider, for the Alloxanthine signal, both what has become of the coupled oxygen atom of the Very Rapid signal and where the coupled nitrogen atom is located. The Alloxanthine signal is unique among Mo(V) e.p.r. signals from xanthine oxidase in failing to show coupling to ^{17}O . In view of the very strong coupling to ^{17}O in the analogous Very Rapid signal, we consider it much more likely that this oxygen atom (whose fate we consider below) is completely missing in the Alloxanthine-signal-giving species than that it is present but coupled too weakly to be detected. With regard to the origin of the coupled nitrogen atom, we suggest, though because of the difficulty of synthesizing the appropriately labelled alloxanthine we have not been able to confirm this proposal by ^{15}N -substitution studies, that the most likely candidate is the one in the 2-position of the alloxanthine molecule (the 2-position being analo-

gous to the 8-position in xanthine, which is the site at which this substrate is oxidized).

In the light of the above points and those considered below, we propose that the structure of the Alloxanthine-signal-giving species is as shown in Fig. 4(b). It is necessary to relate the proposed structure to the known (Massey *et al.*, 1970; Williams & Bray, 1981) kinetics of inhibition by alloxanthine. However, comparison of the structures of the xanthine and alloxanthine complexes in Figs. 4(a) and 4(b) at once raises a serious apparent problem. In Fig. 4(b) molybdenum is shown linked directly to the five-membered heterocyclic ring, whereas in Fig. 4(a) it is shown so linked via an oxygen atom. This raises the question as to whether, with the oxygen atom missing in the complex, as shown, alloxanthine could fit snugly into an active-site pocket that normally accommodates the xanthine molecule. The answer to this question is almost certainly negative, but, for the reasons discussed below, this is probably not important in relation to the inhibition.

Kinetic studies by Williams & Bray (1981) showed that alloxanthine reacts with reduced xanthine oxidase by a two-step mechanism. The first step is rapid and reversible and is characterized by a K_i value of about $1\ \mu\text{M}$ (a value that is of the same order of magnitude as the apparent K_m for xanthine, which is about $5\ \mu\text{M}$). The second

Fig. 4. Proposed structures of (a) the xanthine Very Rapid species (Bray & Gutteridge, 1982) and (b) the Alloxanthine species

Scheme 1. Proposed mechanism for the reaction of alloxanthine with xanthine oxidase

This Scheme is based on the present data as well as on earlier kinetic work (Williams & Bray, 1981). Alloxanthine first reacts rapidly and reversibly with the fully reduced enzyme to form a complex. Water is then eliminated in a slow reaction, causing the N-2 atom of alloxanthine to become ligated to molybdenum(IV). Controlled oxidation leads to the Mo(V) state of this species, showing the Alloxanthine Mo(V) e.p.r. signal. Further oxidation breaks down the complex and liberates the enzyme and inhibitor unchanged.

reaction step is much slower, with a forward rate constant of about 0.006 s^{-1} , and with a reverse rate constant that is almost vanishingly small. It is the rate of this slow second reaction step, which correlates both with generation of the characteristic absorption difference spectrum (λ_{max} 380nm) associated with formation of the alloxanthine complex, and with generation of the Alloxanthine e.p.r. signal.

Combining the kinetic and the e.p.r. data, we therefore propose that the mechanism of reaction with alloxanthine is as shown in Scheme 1. The enzyme is first reduced to the Mo(IV) state, in which both the oxygen and sulphur ligands of molybdenum are expected to be protonated, as shown. In this state the alloxanthine molecule forms a Michaelis complex analogue (K_i about $1\text{ }\mu\text{M}$), in which the inhibitor molecule is presumably orientated, and located in the active-site pocket as for a xanthine molecule, but is not necessarily co-ordinated to molybdenum. The slow and nearly irreversible reaction then takes place. We assume that this involves nucleophilic attack on molybdenum by the alloxanthine nitrogen atom in the 2-position, to displace the oxygen ligand of the metal by loss of a water molecule, and with the alloxanthine molecule, of necessity, in the process moving somewhat nearer to molybdenum within the active-site pocket. The development of an absorption band at 380nm at this stage would be consistent with charge transfer from the nitrogen atom to molybdenum(IV). The complex may then be oxidized slowly (and probably reversibly) by ferricyanide to give the Alloxanthine signal. Over-oxidation results in liberation of alloxanthine and reversion of the enzyme to the oxidized form, with a terminal oxygen and a terminal sulphur ligand (Bordas *et al.*, 1980; Cramer *et al.*, 1981). Our success in maximizing conversion into the signal-giving species is no doubt critically dependent on the relative values of the rate constants for inter-conversion of the Mo(IV), Mo(V) and Mo(VI) species. These rates may well depend both on mediator concentrations and on redox potentials.

All the data are thus consistent in indicating, contrary to earlier assumptions, that alloxanthine, in inhibiting xanthine oxidase, undergoes at the molybdenum centre a very specific reaction that is quite distinct from the normal catalytic reaction with substrates (Bray *et al.*, 1983). The product is a complex that, though it is geometrically very like that of the Very-Rapid-signal-giving intermediate, is nevertheless chemically appreciably different. Further physical studies on the alloxanthine complex would be of particular interest and should be facilitated by its relatively high stability.

These results permit a new classification (Bray *et al.*, 1983) of some of the tightly bound inhibitors of

xanthine oxidase, according to whether these react with the terminal oxygen atom or with the terminal sulphur atom of the oxidized enzyme. Mercurial compounds (George & Bray, 1983b) and arsenite (George & Bray, 1983a) clearly react at the sulphur atom. Aldehydes (Morpeth & Bray, 1984), in inhibiting, appear to react with two ligands, to form cyclic complexes. The ligands could be two oxygen atoms (Bray & Gutteridge, 1982), but might conceivably be one oxygen atom and one sulphur atom. Alloxanthine appears to be unique in reacting only with an oxygen ligand, and eliminating it in the process.

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