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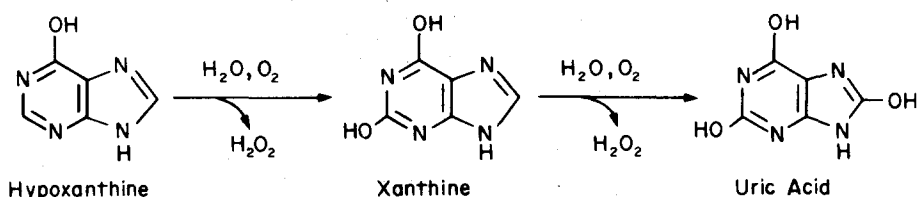
TIGHT BINDING INHIBITORS OF XANTHINE OXIDASE

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1. INTRODUCTION

Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid as follows



The enzyme is well-suited to carry out the catalysis of these oxidative reactions, containing as it does four redox-active sites: molybdenum, flavin adenine dinucleotide (FAD) and a pair of iron-sulfur centers of the ferredoxin type (Fe_2S_2). The purine substrates interact with the enzyme at the molybdenum site of xanthine oxidase and oxygen at the flavin site. The two metabolic reactions above constitute the final steps of purine catabolism in primates and as such play an important role in determining the *in vivo* steady state levels of the various purines, particularly that of uric acid. The introduction of potent inhibitors of xanthine oxidase has proven a clinically effective way to control the hyperuricemia associated with gout (Elion *et al.*, 1963; Duggan *et al.*, 1975; cf. Rundles *et al.*, 1969, for a review) and attenuate the activity of mercaptopurinol and other antileukemic drugs (Rundles *et al.*, 1963). In addition, *in vitro* studies on the mechanism of action of these compounds have shed considerable light on the properties of the enzyme itself (e.g. Massey *et al.*, 1970a,b). This review will concern itself primarily with such *in vitro* studies, and the reader is referred to the review by Rundles *et al.*, for clinical considerations. Two review articles on the general properties of xanthine oxidase have appeared recently (Massey, 1973; Bray, 1975).

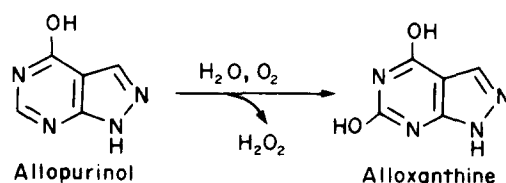
Potent inhibitors of xanthine oxidase can be divided into two broad categories: (1) molecules which are analogs of the purine substrates to a greater or lesser extent and (2) molecules which bear no particular structural relationship to the physiological substrates. The former group consists of pyrazolo[3,4-d]pyrimidines (alloxanthine being the most important among them), pterins such as 6-pteridylaldehyde, and certain other aromatic heterocycles. Included in the latter group are cyanide, arsenite, and methanol, compounds which do not inhibit xanthine oxidase exclusively, but nonetheless interact very specifically with the enzyme. Both classes of inhibitors act at or very near the molybdenum site of the enzyme, or with the active site cyanolysable sulfur atom (cf. Section 3.1), and interfere with the reaction of enzyme with the reducing substrates (hydroxypurines) rather than oxygen. The following discussion will deal first with the purine analogs, about which a good deal is known, and then take up the second class of inhibitors, about which a good deal less is understood.

2. PURINE ANALOGS

2.1. ALLOPURINOL AND ALLOXANTHINE

2.1.1. Mechanism of Action

Pyrazolo[3,4-d]pyrimidines were first reported to be inhibitors of xanthine oxidase by Feigelson *et al.* (1957). Later clinical and enzymological studies by Elion and coworkers established that allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine) was a particularly effective inhibitor *in vivo* as well as *in vitro* (Elion *et al.*, 1963, 1966; Rundles *et al.*, 1963). It was further shown that allopurinol was enzymatically oxidized to the 4,6-dihydroxy compound alloxanthine (Elion *et al.*, 1966). Prior incubation of xanthine oxidase with allopurinol resulted in total loss of activity in xanthine–oxygen oxidoreduction assays, whereas incubation with alloxanthine resulted in no initial inhibition, but progressive inactivation during the course of the assays (Elion, 1966). These findings suggested that inactivation was due to inhibitor binding to an intermediate in the catalytic cycle.



Subsequent work by Massey and coworkers (Massey *et al.*, 1970a,b) established that allopurinol inhibited xanthine oxidase by forming a very tight complex with reduced molybdenum at the active site after having been oxidized by the enzyme to alloxanthine. When xanthine oxidase was incubated with allopurinol under anaerobic conditions, significant reduction of the enzyme was observed spectrophotometrically (Fig. 1, curve 2). On admission of air the enzyme became reoxidized, but the spectrum of the inactive material obtained (Fig. 1, curve 3) differed from that of untreated enzyme (curve 1). An

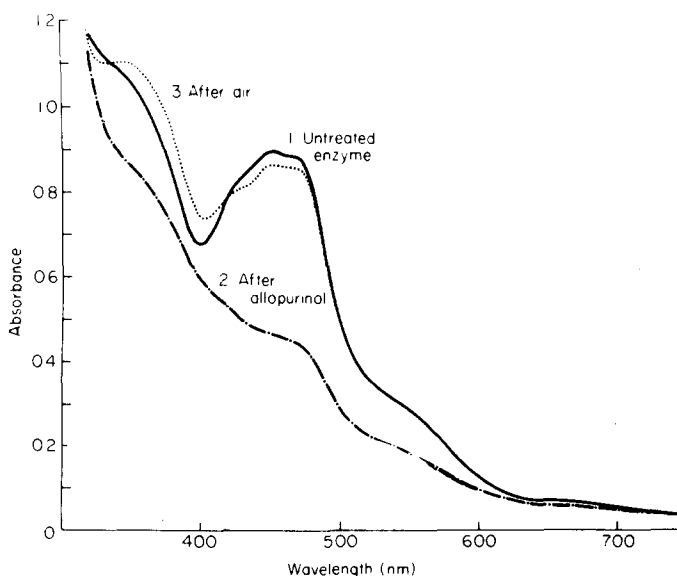


FIG. 1. Effect of allopurinol on the spectrum of xanthine oxidase. Curve 1, untreated enzyme, 2.38×10^{-5} M with respect to FAD, in 0.1 M pyrophosphate pH 8.5, 25°C. Curve 2, spectrum recorded 5 min after the addition of 1.67×10^{-4} M allopurinol anaerobically. The spectrum remained unchanged over a period of 2 hr. Curve 3, immediately after mixing with air; further addition of allopurinol or xanthine anaerobically resulted in no spectral changes over a period of 2 hr (From Massey *et al.*, 1970b).

identical spectrum could be obtained if alloxanthine plus xanthine was used in place of allopurinol, implicating alloxanthine as the actual inhibiting species. In both cases, the difference between the spectra taken before and after admission of air was identical to the difference between the spectra of oxidized enzyme and enzyme reduced by xanthine (Fig. 2). This strongly suggested that the iron-sulfur and FAD chromophores of the enzyme were unaltered and that alloxanthine was interacting at the molybdenum site. Furthermore, when the inhibited reoxidized enzyme was incubated with potassium ferricyanide, an additional two electron equivalents per active site were extracted from the enzyme, resulting in full recovery of activity and a return of the spectrum to that of native enzyme. As the iron-sulfur and FAD sites were oxidized at the outset, this indicated that the molybdenum was still reduced in the inhibitory complex. This inhibitory complex between alloxanthine and reduced molybdenum precludes catalytic reaction involving the molybdenum site, but has no effect on the NADH-ferricyanide activity of xanthine oxidase (Elion, 1966), an activity known to involve only the iron-sulfur and FAD sites of the enzyme (Komai *et al.*, 1969).

Alloxanthine binding was found to be so tight that even at an enzyme concentration of 5×10^{-6} M it bound to the reduced enzyme stoichiometrically (Massey *et al.*, 1970a). This permitted its use in functional site titrations of protein samples and led to the recognition of appreciable amounts (20% or more) of inactive xanthine oxidase in apparently homogenous enzyme preparations. An affinity chromatography technique for resolving active and inactive xanthine oxidase was subsequently developed that relied on the tight binding of pyrazolo[3,4-d]pyrimidines to functional enzyme in the reduced state (Edmondson *et al.*, 1972).

The orientation of the nitrogen atoms within substrate analogs is apparently critical for the expression of this type of xanthine oxidase inhibition. While a series of pyrazolo[3,4-d]pyrimidines all exhibited behavior like that of allopurinol in inhibiting the enzyme (Figs 2 and 3), a group of pyrazolo[4,3-d]pyrimidines, triazolopyrimidines and pyrrolopyrimidines did not (Massey *et al.*, 1970b). These latter compounds were found to be substrates for the enzyme but in no case were spectral perturbations similar to those of the pyrazolo[3,4-d]pyrimidines observed, nor was enzyme inhibition detected.

A key question remaining as regards the mechanism of inhibition of xanthine oxidase by allopurinol and other pyrazolo[3,4-d]pyrimidines has to do with the large number of turnovers (thirty or more) required to effect inhibition during aerobic turnover (Massey

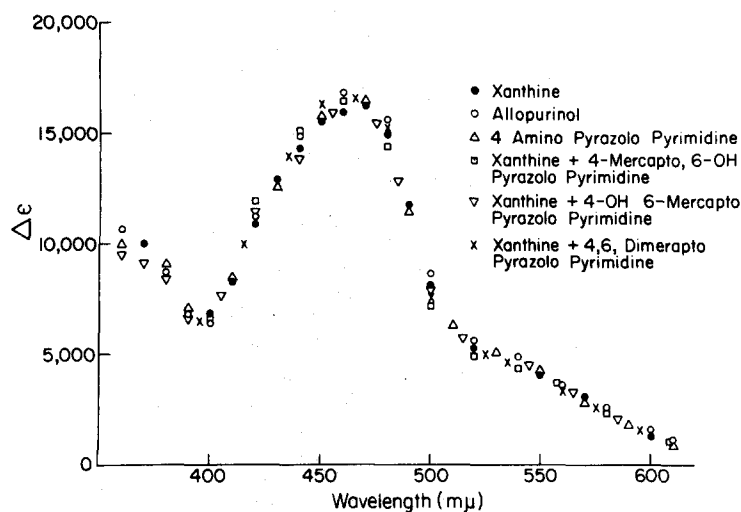


FIG. 2. Difference spectra (expressed as $\Delta\epsilon$ based on concentration of enzyme-bound flavin) between air-reoxidized enzyme and enzyme reduced as indicated. With xanthine as reductant the difference calculated was between oxidized enzyme and the spectrum produced immediately after addition of xanthine. The difference spectra obtained with the pyrazolo[3,4-d]pyrimidines was, for example, that between curves 3 and 2 of Fig. 1 (from Massey *et al.*, 1970b).

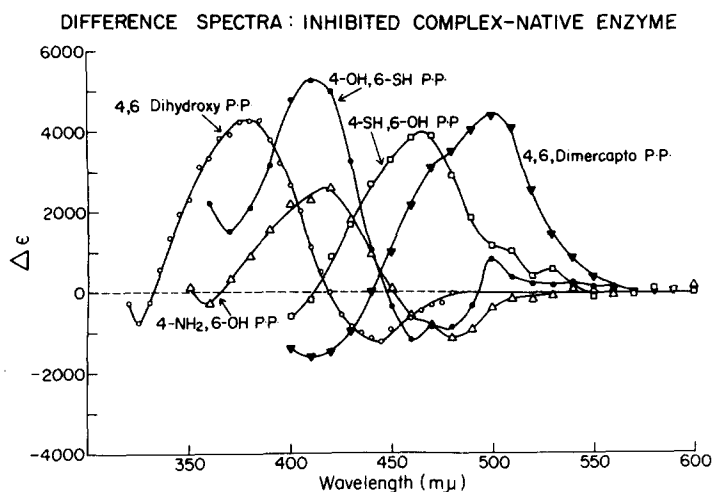


FIG. 3. Difference spectra between xanthine oxidase inactivated with various pyrazolo[3,4-d]pyrimidines and native enzyme. The spectra represent, for example, the difference between curves 3 and 1 of Fig. 1.

et al., 1970a; Spector and Johns, 1970). Why is the enzyme-product complex of alloxanthine with reduced enzyme which is formed in the first turnover unable to prevent subsequent turnovers? Clearly the dissociation constant for the first several alloxanthine molecules formed must be much greater than that for alloxanthine participating in the ultimate inhibitory complex. A clue lies in the observation that, along with a pair of electrons, a proton is transferred from substrate to molybdenum in the early steps of catalysis (Edmondson *et al.*, 1973). Thus the initial complex on formation of product would be alloxanthine · Mo^{IV} · H⁺. The likely sequence of events in the decay of this species is alloxanthine dissociation followed by redistribution of electrons from molybdenum to iron-sulfur and flavin sites, and proton dissociation. Subsequent rebinding of alloxanthine to the molybdenum site of fully reduced enzyme would give rise after a number of turnovers to a complex not containing a proton, and conceivably having a much lower K_a for alloxanthine. This scheme, while highly speculative, provides a mechanistically reasonable explanation for the observed delay in the onset of inhibition by allopurinol.

2.1.2. Clinical Considerations

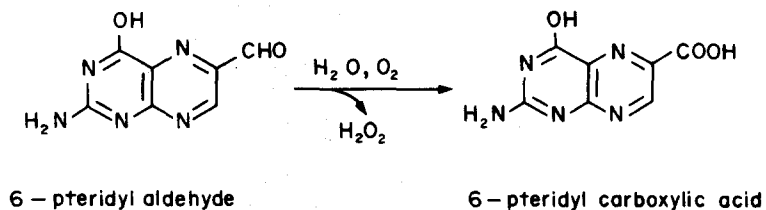
Allopurinol has been found to be a clinically effective, well-tolerated drug in the treatment of hyperuricemia associated with gout (Elion *et al.*, 1963; Rundles *et al.*, 1969; Yu and Cutman, 1964). Doses as high as 700–1000 mg per day can be administered for extended periods in relative safety to maintain serum and urinary urate levels well below the limit of solubility of 7 mg% (Rundles *et al.*, 1963; Goldfinger, 1971). Significantly, allopurinol does not appear to induce synthesis of xanthine oxidase or to shorten its *in vivo* half-life, nor does it increase the steady state levels of the various purine pools (Rundles *et al.*, 1969; Goldfinger, 1971). Side-effects are rare, but include leukopenia, thrombocytopenia, renal impairment, dermatitis, rashes and mild gastrointestinal disorders (Rundles *et al.*, 1969; Chalmers *et al.*, 1969). In general, it appears that withdrawal of treatment results in amelioration of symptoms when they occur.

It has been established however, that allopurinol can condense with phosphoribosyl-pyrophosphate in a reaction catalyzed by IMP-pyrophosphate phosphoribosyltransferase to form a nucleotide with anti-metabolic potential (McCollister *et al.*, 1964; Chalmers *et al.*, 1969). Conversion to the nucleoside by purine nucleoside phosphorylase has also been reported (Krenitsky *et al.*, 1967). Because the effects of these compounds are unknown and could well be deleterious after long periods of time, efforts to find new drugs

equally potent as inhibitors of xanthine oxidase but lacking reactivity in these undesirable side reactions have been made.

2.2. OTHER SUBSTRATE ANALOG INHIBITORS OF XANTHINE OXIDASE

Historically, the first potent inhibitor of xanthine oxidase discovered was a breakdown product of folic acid (Kalckar and Klenow, 1948a,b), later identified as 2-amino-4-hydroxy-6-formylpteridine, commonly known as 6-pteridylaldehyde (Kalckar *et al.*, 1948). Lowry *et al.* (1948) determined the K_i for this compound to be 6×10^{-10} M and showed that it was slowly converted by the enzyme to 6-pteridyl carboxylic acid.



In at least one instance (Oster, 1976) folic acid has been mistakenly used clinically to treat hyperuricemia, the effective agent presumably being 6-pteridylaldehyde produced from catabolism of folic acid (or present as an impurity in the folic acid to begin with). Recent studies by Kaplan (1980) indicate that folic acid itself is probably not an effective inhibitor of xanthine oxidase.

The reaction of 6-pteridylaldehyde with xanthine oxidase may be followed kinetically by observing the increase in long-wavelength absorbance ($\lambda_{\max} = 620$ nm) on binding of inhibitor to enzyme (Fig. 4). Results from this laboratory (Massey, unpublished results)

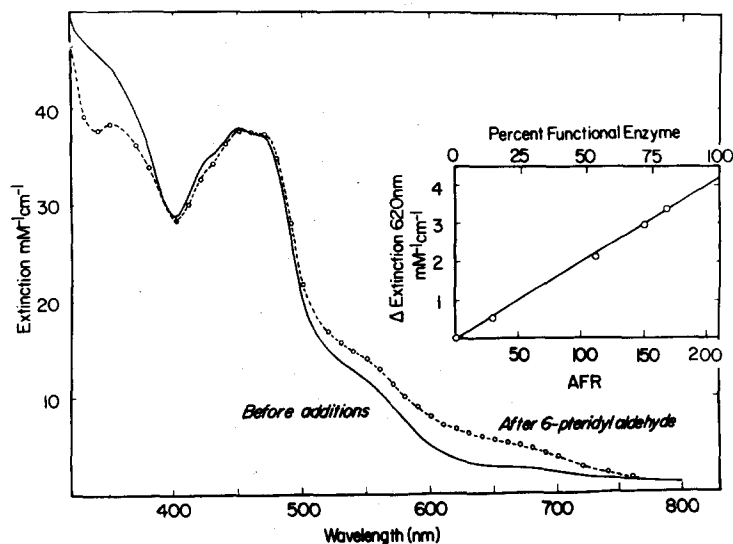
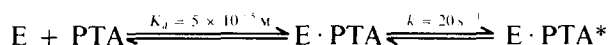


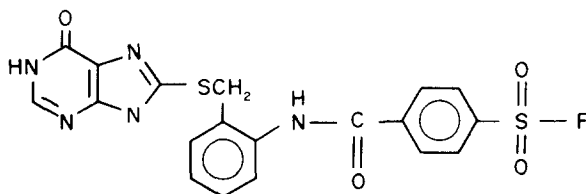
FIG. 4. The effect of 6-pteridylaldehyde on the spectrum of xanthine oxidase. Solid line; native enzyme, in 0.1 M pyrophosphate pH 8.5, 25°C. Dashed line; after the addition of 6-pteridylaldehyde, aerobically. Inset; the relationship between the extinction change observed at 620 nm on addition of 6-pteridylaldehyde to the fraction of active enzyme in solution. AFR represents the activity-to-flavin ratio, a measure of functional enzyme in solution. It is defined as the change in absorbance per min at 295 nm in a 3 ml reaction volume divided by the absorbance at 450 nm of the xanthine oxidase used in the assay, under the standard conditions of 100 μ M xanthine, 250 μ M O_2 , in 0.1 M pyrophosphate, pH 8.5, 25°C.

indicate that the species giving the long wavelength absorption arises from a pre-equilibrium having a K_d of 5×10^{-5} M and appears at a limiting rate of 20 s^{-1} at high pteridylaldehyde concentrations (Fig. 5)



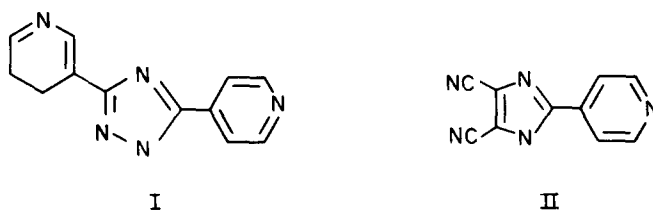
where $E \cdot \text{PTA}^*$ represents the absorbing species. A rate on the order of $2 \times 10^{-3} \text{ s}^{-1}$ for the reverse reaction of the second step would account for the observed overall K_i of 10^{-9} M. The amount of absorbance change observed on addition of pteridylaldehyde to enzyme is directly proportional to the specific activity of the enzyme. Prior treatment with cyanide, known to chemically alter the active site by removing an essential sulfur atom as SCN^- (cf. Section 3.1.), is found to prevent the appearance of the long wavelength absorption. This in conjunction with the observation that the same absorbance change was observed on binding of 6-pteridylaldehyde to deflavo enzyme (eliminating the possibility of flavin radical as the absorbing species) suggests involvement of the molybdenum site (or the cyanolysable sulfur) in the structure of the chromophore. Binding of 6-pteridylaldehyde to xanthine oxidase is so tight that substoichiometric amounts of inhibitor were found to inhibit the enzyme completely, thus providing the first evidence for the existence of nonfunctional forms of xanthine oxidase (Lowry *et al.*, 1948). It is possible that the long-wavelength absorbing species represents a transient covalent intermediate in the catalytic cycle (cf. Fig. 6, intermediate II), dramatically stabilized in the case of 6-pteridylaldehyde (Davis, 1980).

Pursuing another line, Baker and Wood (1967) showed that 9-phenylguanine inhibited xanthine oxidase some 140 times more effectively than did 9-methylguanine and that the aromatic interaction with enzyme was hydrophobic rather than charge-transfer in nature. Further investigation into this hydrophobic interaction led to the tailoring of active-site directed irreversible inhibitors of xanthine oxidase (Baker and Wood, 1968a,b; Baker *et al.*, 1968). These compounds contained good leaving groups on the aromatic substituents, rendering them susceptible to nucleophilic attack by basic amino acid residues near the active site of the enzyme and leading to covalent modification of the protein. Among the most effective of these inhibitors was the following compound



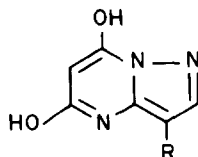
This compound was found to exhibit an I_{50} of 10^{-7} M (under the assay conditions of $8 \mu\text{M}$ xanthine and $250 \mu\text{M}$ oxygen) and greater than 80% covalent inhibition after 20 min of incubation. The mode of covalent attachment to the enzyme was not determined in these studies, but on the basis of the current understanding of the enzyme mechanism (Fig. 6), it is tempting to speculate that the nucleophile at the enzyme active site is a persulfide residue (cf. Section 3.1.) important in the catalytic cycle. The clinical usefulness of these compounds is uncertain due to likely interactions of these compounds with other enzymes involved in purine metabolism (Baker and Wood, 1968a,b) and possible negative effects on the *in vivo* stability of xanthine oxidase as a result of covalent modification.

Recently a comprehensive survey of potential inhibitors of xanthine oxidase among substituted imidazoles and triazoles was undertaken by Baldwin *et al.* (1975, 1977). Typical of the compounds found most effective in *in vitro* enzyme assays were 3-(3-pyridyl)-5-(4-pyridyl)-1,2,4-triazole (I) and 2-(4-pyridyl)-4,5-dicyanoimidazole (II). Both of



these compounds were some 100-fold more effective than allopurinol, with I_{50} values at $300 \mu\text{M}$ hypoxanthine and $250 \mu\text{M}$ oxygen of $2 \times 10^{-8} \text{ M}$, compared with I_{50} for allopurinol of $3 \times 10^{-6} \text{ M}$. Among the triazoles tested it was found that 4-pyrimidinyl, 4-pyridyl, 5-quinolyl or several halogenated benzene derivatives could be substituted for the 3-pyridyl residue at position 3 without significant loss of inhibitory capacity, but substitution of 2-pyridyl or 2-pyrimidinyl resulted in a thousand-fold decrease in I_{50} (Baldwin *et al.*, 1975). As was the case with the pyrazolo[3,4-d]pyrimidines, the relative positions of the nitrogen atoms within the heterocycles are apparently critical for the expression of inhibitory activity. In the imidazole series, nitro or carboxyl groups were acceptable substitutes for the cyano group at positions 4 and 5 (resulting in only a slight loss of inhibition) but amido or carboxymethyl groups were not. For both imidazoles and triazoles, a pyridyl function and a free imino group in the five-membered ring were required for inhibitory activity. These compounds were apparently not hydroxylated by xanthine oxidase (Duggan *et al.*, 1975).

Springer *et al.* (1976) reported the inhibition of xanthine oxidase by 5,7-dihydroxypyrazolo[1,5- α]pyrimidines having aromatic substituents at position 3



The most potent of these inhibitors, with R = benzyl, 3-pyridyl or 1-naphthyl, exhibited K_i s 50-fold less than allopurinol ($2 \times 10^{-8} \text{ M}$, versus 10^{-6} M for allopurinol). These workers compared this with a K_i for allopurinol of 10^{-6} M , but 10^{-10} M appears to be a better estimation of that value (Spector and Johns, 1970). Inhibition was found to be of

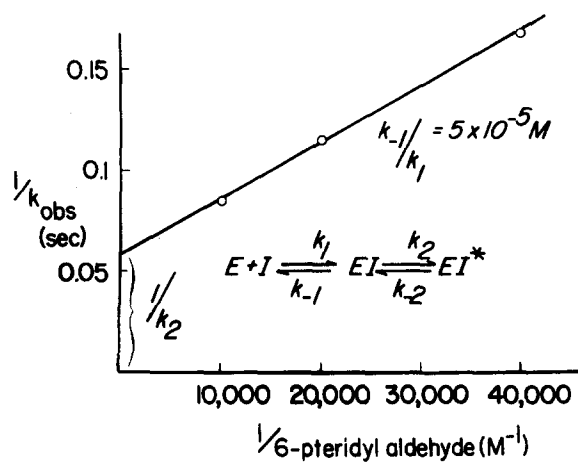


FIG. 5. The dependence of the rate of formation of the long wavelength absorbance from Fig. 4 on 6-pteridylaldehyde concentration. From the intercept and slope of the double reciprocal plot values of 20 s^{-1} for k_2 and $5 \times 10^{-5} \text{ M}$ for k_{-1}/k_2 can be determined, respectively.

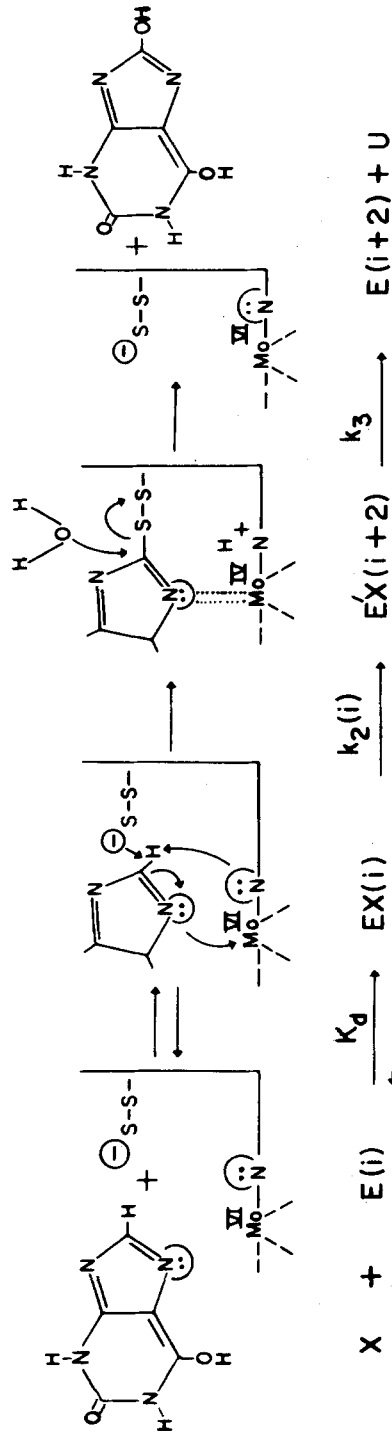


FIG. 6. A model for the mechanism of action of xanthine oxidase making use of the proposed persulfide residue at the active site. The persulfide initiates catalysis by nucleophilic attack at C₈ of xanthine, followed by hydrolysis of the covalent intermediate to give uric acid and enzyme reduced by two electrons. (From Olson *et al.*, 1974).

the non-competitive variety, although as Cha points out (Cha, 1975) apparently non-competitive inhibition is to be expected regardless of the nature of the enzyme-inhibitor interaction, given the very tight binding of these inhibitors.

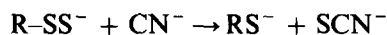
In conducting studies of this general nature, two considerations must be kept in mind. First, I_{50} values reflect only an apparent K_i at the given substrate concentrations used in the assay. In the absence of additional information it is impossible to extrapolate such data to obtain values for the true K_i , the parameter that accurately reflects the affinity of the inhibitor for the enzyme to which it binds. Cha has discussed this and other problems associated with the study of tight-binding inhibitors in general (Cha, 1975) and the interaction of allopurinol and alloxanthine with xanthine oxidase in particular (Cha *et al.*, 1975). In brief, the relationship of I_{50} to K_i is shown to depend on the type of inhibition and may be further complicated by failure to achieve a steady-state condition in the course of enzyme assays. The second consideration is that all comparisons of prospective drugs seem to be made with allopurinol. In fact, alloxanthine (the product of enzyme oxidation of allopurinol) is the actual inhibitor, and its dissociation constant with reduced enzyme is about 10^{-10} M (Massey *et al.*, 1970a; Spector and Johns, 1970), considerably less than the I_{50} value of 10^{-6} M for allopurinol used for comparative purposes. Such being the case, the apparent attractiveness of many inhibitors becomes greatly diminished when compared to alloxanthine.

3. OTHER INHIBITORS OF XANTHINE OXIDASE

3.1. CYANIDE

Cyanide was first shown to inhibit xanthine oxidase by Szent-Gyorgyi (1926). Fridovich and Handler (1958) reported that $^{14}\text{CN}^-$ bound to the enzyme with a stoichiometry of one molar equivalent per active site, but it was subsequently shown by Massey and Edmondson (1970) that this was not the source of inactivation. Rather, cyanide reacted with the enzyme to release an essential sulfur atom as thiocyanate. Activity could be recovered to a significant extent by incubation with Na_2S . When Na_2^{35}S was used, a second treatment with CN^- produced $^{35}\text{SCN}^-$. Using an affinity column procedure based on the tight binding of pyrazolopyrimidines to reduced enzyme, Edmondson *et al.* (1973) were able to separate active and inactive xanthine oxidase, and show that the inactive enzyme found in standard preparations was identical to the cyanide-treated, or desulfo, species.

On the basis of the chemical evidence, Massey and coworkers (Massey and Edmondson, 1970; Olson *et al.*, 1974) have proposed that xanthine oxidase contains a persulfide group at its active site. This persulfide is postulated to act as a nucleophile in the course of catalysis, as shown in Fig. 6, and be susceptible to cyanolysis in a reaction typical of persulfides

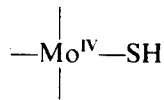


In order to explain the observation that treatment with cyanide under anaerobic conditions results in enzyme reduction, a nearby thiol is proposed that spontaneously forms a disulfide with the product of cyanolysis (Massey and Edmondson, 1970). This second thiol would also play a part in the binding of arsenite, another inhibitor of xanthine oxidase (see below). It is known, however, that fully reduced enzyme is not inactivated by cyanide (Fridovich and Handler, 1958), presumably due to a compulsory transfer of electrons to Mo in the course of inactivation. Disulfide formation would therefore have to be an obligatory step in cyanide inactivation according to the persulfide scheme. There appears to be no compelling reason why this must be so. In addition, the essential sulfur site exhibits certain properties difficult to reconcile with persulfides. For example, it is stable at low pH, as evidenced by the fact that native and desulfo enzyme release the same amount of acid-labile sulfur (Massey and Edmondson, 1970), and is

unreactive with nucleophiles such as hypotaurine, to which the active site should be accessible.

Two alternate proposals regarding the chemical nature of the essential sulfur site have been put forward recently. Coughlan (1977) has proposed a cysteinyl residue in complex with molybdenum, which is susceptible to nucleophilic attack by CN^- (Fig. 7A). The scheme accounts for the observed stoichiometry of one thiocyanate produced per active site, the generation of reducing equivalents in the course of inactivation, and at first glance, the known fixation of CN^- to the enzyme (Fridovich and Handler, 1958; Coughlan *et al.*, 1969). However, the scheme predicts one CN^- fixed for each SCN^- produced. This is not in agreement with results of Massey and Edmondson (1970) which showed that after one hour in the presence of 9 mM K^{14}CN , the enzyme was fully inactivated and the stoichiometry of S^{14}CN^- released per active site was 0.96:1.0, but the amount $^{14}\text{CN}^-$ fixed to protein was only 0.39:1.0. In addition, to explain the observation of naturally occurring inactive enzyme, and its activation by Na_2S , the highly speculative scheme in Fig. 7B must be proposed. Apart from the uncertain chemistry, it is not clear that the dehydroalanine and cyanoalanine species proposed for naturally occurring and cyanide inactivated enzyme forms, respectively, would be expected to give the same absorption spectra, as is observed (Edmondson *et al.*, 1972). Also, as was the case with the persulfide model, there is no clear reason from this scheme why prior reduction of the molybdenum should prevent inactivation.

A third proposal has been considered by Bray and coworkers (Gutteridge *et al.*, 1978; Bray *et al.*, 1979). In this model the essential sulfur atom exists as a terminal sulfo ligand to the molybdenum. Reaction of this species with CN^- would follow the scheme shown in Fig. 8 and account for the SCN^- and redox equivalent stoichiometry that is observed experimentally. Prior reduction of the molybdenum site would probably give rise to a species with the following structure, due to the large shifts of pK of Mo ligands generally associated with changes in redox state of molybdenum (Steifel, 1977)



This species would not be expected to be susceptible to CN^- because, on the basis of the model, Mo would have to be reduced beyond the IV valence state in the course of inactivation. Electron paramagnetic resonance data provide further evidence, albeit somewhat indirect, in favor of this model. Both native and desulfo xanthine oxidase exhibit Mo^{V} signals strongly coupled to protons, but on dilution into D_2O the proton of the desulfo enzyme exchanges some 200-fold more slowly than the proton of the native enzyme. Assuming proton association is diffusion-controlled for both forms of the

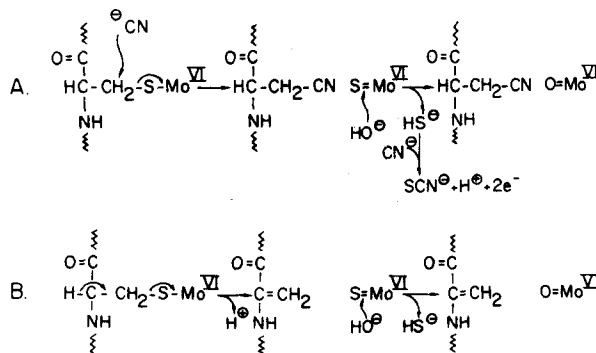


FIG. 7. A model for the active site of xanthine oxidase based on cysteine as the source of cyanolysable sulfur. A: scheme proposed for the cyanolysis reaction. B: scheme proposed to account for naturally occurring inactive enzyme (from Coughlan, 1977).

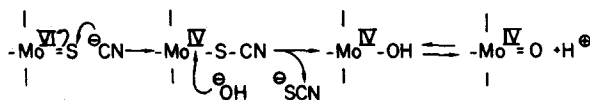


FIG. 8. Scheme for the cyanolysis of a Mo = S complex.

enzyme, the above difference in rates of dissociation corresponds to a difference in pK of 2, with native enzyme being more acidic. This is of the order expected if the protons were binding directly to sulfo and oxo ligands in native and desulfo enzyme, respectively, given the greater acidity of sulfur-bound protons compared to oxygen-bound ones (Gutteridge *et al.*, 1978; Steifel, 1977). Similarly, the much lower redox potential of the molybdenum site of desulfo xanthine oxidase compared to that of native enzyme (-460 mV vs -355 mV, respectively; Cammack *et al.*, 1976) may be held as consistent with the substitution of oxygen for sulfur in the desulfo species. Recent data from extended X-ray absorption fine structure (EXAFS) experiments support the notion that the sulfur lies in the coordination sphere of molybdenum (Bordas *et al.*, 1980). In this method, the attenuation of X-ray absorption due to the presence of nuclei in the vicinity of the absorbing nucleus (in this case molybdenum) is simulated empirically using parameters obtained from studies on model compounds of known three dimensional structure. EXAFS has proven extremely sensitive to the type of atom and its distance from the absorbing nucleus, and a comparison of the EXAFS spectra for native and desulfo xanthine oxidase clearly indicates a difference in the molybdenum environment of the two enzyme forms. Unfortunately, while this difference reflected changes in xanthine oxidase coordination at a distance consistent with those expected for the conversion of Mo=S to Mo=O, simulations were not able to demonstrate unambiguously the loss of a sulfur atom and the gain of an oxygen atom in the coordination sphere of xanthine oxidase on treatment with cyanide. In a comparative study of the EXAFS spectra of sulfite oxidase and xanthine oxidase with a series of model compounds (Berg *et al.*, 1979; Tullius *et al.*, 1979) it was found that sulfite oxidase yielded a spectrum virtually identical to that of the compound shown in Fig. 9. Xanthine oxidase gave a somewhat different, although still very similar EXAFS spectrum. The xanthine oxidase sample used in these studies, however, was not fully active and therefore heterogeneous as regards the molybdenum center. The EXAFS spectrum of the desulfo form (Bordas *et al.*, 1980) appears to resemble more closely that of the model compound (Tullius *et al.*, 1979) although direct comparison of spectra from the two references is difficult.

The observation most difficult to explain with the Mo=S model comes from experiments using a *Neurospora* mutant that synthesizes aponitrate reductase but not apparently, an essential molybdenum cofactor (Ketchum *et al.*, 1970; Nason *et al.*, 1971). Rajagopalan and coworkers have reported that the molybdenum cofactor isolated by acid treatment from cyanide-treated as well as native xanthine oxidase is capable of restoring nitrate reductase activity to the *Neurospora* apoenzyme (Hainline *et al.*, 1979). This result is inconsistent with the notion that the essential sulfur is an integral part of a molybdenum cofactor as it must be if it exists as Mo=S. It may be possible, however, to

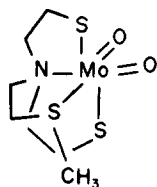


FIG. 9. The structure of $\text{MoO}_2[(\text{SCH}_2\text{CH}_2)_2\text{NCH}_2\text{CH}_2\text{SCH}_3]$, a model for the active site of xanthine oxidase.

reconcile these results with the Mo=S model. Neither sulfite oxidase (which can also provide molybdenum cofactor active in the *Neurospora* reconstitution experiment) nor nitrate reductase appear to be inhibited by cyanide in the same way as xanthine oxidase; i.e. by releasing sulfur in the form of thiocyanate. It is conceivable that Mo=O, formed spontaneously from Mo=S in the acid extraction step in the case of native xanthine oxidase and occurring naturally in the case of sulfite oxidase, is the species responsible for the reconstitution of nitrate reductase activity and that the sulfur needed for xanthine oxidase activity is not required. While this would offer an explanation for the striking similarity in the ESAFS spectra of sulfite oxidase and desulfo xanthine oxidase to the model compound of Fig. 9, and the slight difference in the case of native xanthine oxidase, more work is clearly needed. It is difficult to explain, for example, the well-established inhibition of cyanaolysis by arsenite (Rajagopalan and Handler, 1964; Massey and Edmondson, 1970) in terms of the Mo=S model. The only known mechanism of action of arsenite is to complex very tightly to dithiols, and the extent to which Mo=S would exhibit thiol character is unclear. While a prototropic equilibrium such as discussed above might be invoked to generate Mo^{IV}-SH, this species, if formed, does not react with Kenyon's reagent [methylmethanethiosulfonate (Smith *et al.*, 1975)] in the pH range 7-10 to result in loss of activity (R. Hille, unpublished data), nor with DTNB (Massey *et al.*, 1970a) and so cannot be considered to have the general properties of a thiol.

3.2. METHANOL AND FORMALDEHYDE

Methanol inhibits xanthine oxidase when incubated in the presence of xanthine (Rajagopalan and Handler, 1967; Coughlan *et al.*, 1969) and gives rise to a characteristic EPR signal attributable to Mo^V (Aleman-Aleman *et al.*, 1965). This signal, termed 'Inhibited' by Bray and Vanngard (1969), exhibits a doublet structure indicating that molybdenum is coupled to a single proton. The Inhibited signal generated by using C²H₃OH does not show this doublet structure even when incubated in H₂O for prolonged periods, indicating that the proton coupled to molybdenum in the signal-giving species is derived from the methanol itself and is not exchangeable with the bulk solvent (Pick *et al.*, 1971). Dialysis at high temperatures or extremes in pH, or incubation with Na₂S₂O₄ or K₃Fe(CN)₆ causes the Inhibited molybdenum V signal to disappear and at least partially restores activity. Unlike the other EPR signals of xanthine oxidase, the Inhibited signal is air stable.

Formaldehyde also produces the Inhibited signal with xanthine oxidase, and is able to do so in the absence of added xanthine (Pick *et al.*, 1971). It is likely that methanol is converted to formaldehyde prior to its inactivation of the enzyme. Like methanol, formaldehyde is capable of reducing xanthine oxidase (Booth, 1938; Bray and Meriweather, 1966; Pick and Bray, 1969). Attack by persulfide (or any good nucleophile) on the carbonyl carbon of formaldehyde, in a reaction analogous to the first step of Fig. 6, might give rise to a structure with features consistent with the data (Fig. 10). This involvement of the persulfide would be in agreement with the observation that NC⁻ and formaldehyde are mutually exclusive in their actions on the enzyme (Edmondson *et al.*, 1972). Whatever the structure of the inactivated enzyme, it must provide sufficient stabilization of Mo^V to account for the air-stability of the Inhibited EPR signal. This could be

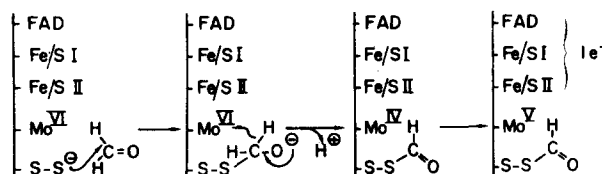


FIG. 10. Scheme proposed for the reaction of xanthine oxidase with formaldehyde giving rise to the enzyme species characterized by the Inhibited Mo^V EPR signal.

accomplished by raising the redox potential of $\text{Mo}^{\text{VI}}\text{-Mo}^{\text{V}}$ couple and/or lowering that of the flavin, where reaction with oxygen takes place.

Ethylene glycol (Tanner and Bray, 1978a,b) and mercaptoethanol (R. Hille, unpublished results) also inhibit xanthine oxidase and are capable of transferring reducing equivalents to it. The former has been shown to be converted to glyoxylate and oxalate by the enzyme (Tanner and Bray, 1978) and give rise to an EPR signal identical to the Inhibited signal except for the absence of doublet structure. This absence of splitting is consistent with the observation that the products expected to be involved in inactivation, glyoxylate and/or oxalate, contain only one carbonyl proton, which would be lost in the course of inactivation.

3.3. ARSENITE

Inhibition of xanthine oxidase by arsenite was first reported by Mackler *et al.* (1954). It was also studied briefly by Peters and Sanadi (1961). The importance of the arsenite inhibition to our understanding of the mechanism of xanthine oxidase did not become clear until Coughlan *et al.* (1969) reported that arsenite treatment, like cyanide, resulted in a spectral change of the enzyme which was coincident with the development of inhibition. They also made the important finding that cyanide and arsenite inhibition are mutually exclusive; prior reaction with cyanide prevents the appearance of the spectral changes typical of arsenite inhibition, and conversely, prior complexing with arsenite severely interferes with cyanide inactivation. The important observation was also made that whereas cyanide inactivation appeared to be irreversible (but see earlier section of cyanide inactivation), the effect of arsenite was reversible by dilution, e.g. by dialysis. They noted also that while the inhibition of xanthine oxidase by arsenite was a slow process, the closely related enzyme, liver aldehyde oxidase, was inhibited immediately (< 10 sec) by 10^{-4} M arsenite, and that this inhibition was reversed rapidly on dilution. Thus they concluded that the effects of arsenite and cyanide were mutually exclusive, and that they both interacted with the enzyme bound molybdenum. As described in the previous section, this concept was discredited by the discovery of the cyanolysable sulfur in this group of enzymes, and the demonstration that this particular sulfur atom was essential for normal catalysis (Massey and Edmondson, 1970). The mutually exclusive reactions of cyanide and arsenite were, however, confirmed, and it was proposed that the common factor was their interaction with the cyanolysable sulfur atom. This was shown experimentally by the linear relationship between catalytic activity, cyanolysable sulfur and the extinction change at 380 nm due to complex formation with arsenite. The same relationship has also been found to exist for the ability to bind rapidly alloxanthine and other pyrazolo[3,4-d]pyrimidines, and for rapid reduction of the enzyme by substrate (Edmondson *et al.*, 1972). The great importance of the cyanolysable sulfur atom to the properties of the enzyme were thus immediately obvious. Of the various hypotheses put forward to account for the chemical nature of this sulfur atom, none is entirely satisfactory. Only that proposed by Massey and Edmondson (1970) deals straightforwardly with the inhibition by arsenite in a way with known precedents, i.e. the strong complexing of arsenite with vicinal thiols. However, a point which has perhaps not received the attention it deserves, is the *slowness* of the reactions of cyanide and arsenite with milk xanthine oxidase (Coughlan, *et al.*, 1969; Massey and Edmondson, 1970). This suggests that the form(s) of the enzyme reacting with these reagents may be minor equilibrium species. In this respect it is intriguing that aldehyde oxidase reacts rapidly with both cyanide and arsenite (Coughlan *et al.*, 1969) and that all preparations so far reported show substantial amounts of a Mo^{V} EPR signal in the resting enzyme (Rajagopalan *et al.*, 1968).

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