Enzyme Systems that Metabolise Drugs and Other Xenobiotics.
Edited by Costas Ioannides

ISBNs: 0-471-894-66-4 (Hardback); 0-470-84630-5 (Electronic)

5 Molybdenum Hydroxylases

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Introduction

Aldehyde oxidase (AO) and xanthine oxidase (XO) are complex molybdoflavoproteins with similar composition and catalytic properties but which differ in their substrate/inhibitor specificities. Xanthine dehydrogenase (XDH) is thought to be a different functional form of XO, coded by the same gene, which reacts preferentially with NAD^+ in contrast to XO and AO which utilise molecular oxygen (O_2) as an oxidising substrate. In mammals XDH is thought to predominate *in vivo,* but this form undergoes a facile conversion to XO during purification via sulphydryl oxidation or proteolysis (Nishino 1994).

Both proteins show a remarkable degree of sequence identity and Terao *et al*. (1998) have suggested that AO and XO are members of a multigene family, which have originated from a relatively recent duplication event. However, evidence to date indicates that there are fewer members of the 'molybdenum hydroxylase' family than those of the cytochrome P450 superfamily. Nevertheless, the wide range of drugs, xenobiotics and endogenous chemicals that interact with these enzymes, particularly AO, highlight the importance of these enzymes in drug oxidation, detoxication and activation (Beedham 1985, 1987, 1997, 1998).

FUNCTIONALISATION REACTIONS CATALYSED BY MOLYBDENUM HYDROXYLASES

Molybdenum hydroxylases catalyse both oxidation and reduction reactions although the prevalence of the former reactions significantly outweighs the latter *in vivo*. Unlike cytochrome P450, oxidative hydroxylation catalysed by the molybdenum hydroxylases generates reducing equivalents and, although both enzymes utilise molecular oxygen (O_2),the ultimate source of the oxygen atom inserted into substrates is water and not O_2 (Xia *et al*. 1999). In most cases, AO and XO have complementary substrate specificities with microsomal monooxygenases.

With respect to substrates, oxidation involves nucleophilic attack at an electron-

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deficient carbon with the insertion of an oxygen atom to generate either a cyclic lactam or a carboxylic acid from aromatic *N*-heterocycles and aldehydes respectively (Figure 5.1). *N*- or *S*-oxidation has not been reported.

In contrast, *N*- and *S*-functional groups are reduced by both AO and XO under anaerobic conditions. Reactions include reduction of *N*-oxides, sulphoxides, hydroxamic acids, epoxides and reductive ring cleavage some of which may also be catalysed by cytochrome P450 reductase.

ENZYME STRUCTURE

AO and XO have unusually broad and overlapping substrate specificities indicative of a relatively flexible or accessible binding site. Both enzymes are homodimers, with each subunit containing a molybdopterin cofactor, FAD and two different 2Fe:2S clusters. Insight into the structural organisation of the proteins has been obtained from the crystal stucture of the related molybdo-enzyme, *Desulphovibrio gigas* aldehyde oxidoreductase (Romão *et al*. 1995). The molybdopterin cofactor, initially characterised by Rajagopalan and Johnson (1992) and later confirmed by the crystal structure of *gigas* aldehyde oxidoreductase contains mononuclear molybdenum (Mo) coordinated to the enzyme via a *cis* dithiolene moiety (Romão *et al*. 1995; Rajagopalan 1997). In addition, Mo is also coordinated to sulphido and oxo ligands in XO and AO with one further ligand, which is either a further oxo ligand, water ligand or probably a metal-coordinated hydroxide (Mo–OH, Figure 5.2) (Xia *et al*. 1999).

REACTION MECHANISM

The overall reaction mechanism can be considered as coupled reductive and oxidative half-reactions; hence different electroactive substrates may undergo oxidation or reduction (Kisker *et al*. 1997). Furthermore, oxidation of reducing substrates such as 2-pyrimidinone can provide the reducing equivalents for numerous reduction reactions under anaerobic conditions (see below). Reducing substrates react at the molybdenum centre via a two-electron redox reaction during which the Mo is reduced from Mo (VI) to Mo (IV) (Kisker *et al*. 1997). The most recent mechanism proposed for the reductive half-reaction of XO is shown in Figure 5.2 (Xia *et al*.1999). Base-assisted hydroxylation at an electron deficient carbon via Mo–OH is thought to precede hydride transfer to the sulphido ligand of Mo. Although water is the ultimate source of oxygen atom incorporated into product, it is proposed that the catalytically labile site is Mo–OH although alternative mechanisms invoke either an oxo-ligand or a buried water molecule in the protein as the attacking species (Hille and Sprecher 1987; Howes *et al*. 1996; Bray and Lowe 1997). In all cases, enzyme turnover would generate active enzyme via reaction with solvent (Hille and Sprecher 1987).

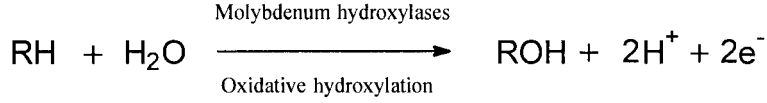


Figure 5.1. Oxidative hydroxylation catalysed by the molybdenum hydroxylases, AO and XO.

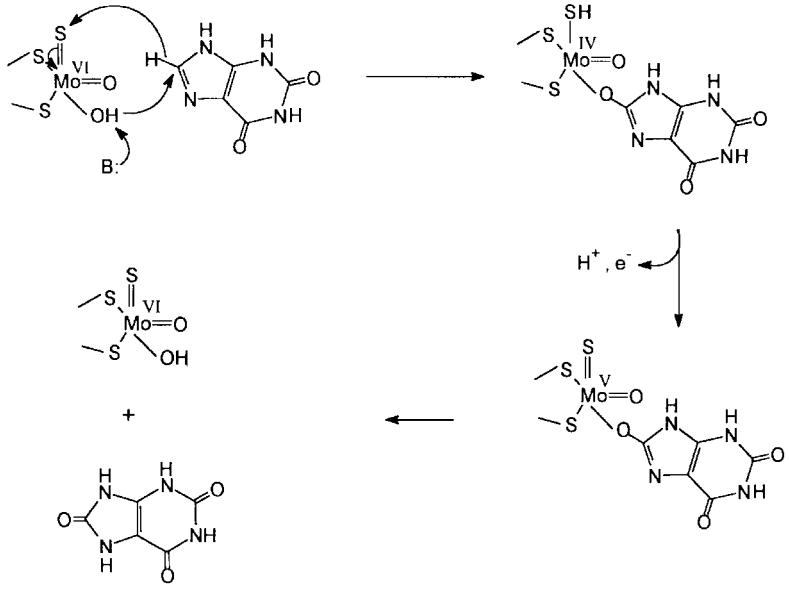


Figure 5.2 Reaction mechanism for the oxidation of xanthine to uric acid catalysed by XO via base-assisted nucleophilic attack (Xia *et al.* 1999).

During the oxidative half-reaction Mo(IV) is re-oxidised via rapid one-electron transfer to an iron-sulphur cluster, generating an intermediate electroparamagnetic Mo(V), and further intramolecular electron transfer to FAD.

RE-OXIDATION OF REDUCED ENZYME

Although oxidising substrates can interact at any of the redox centres, physiological electron acceptors react with the FAD site in XO, XDH and AO (Hille and Nishino 1995). AO and XO generate the reactive oxygen species (ROS), H_2O_2 and O_2^-, during substrate oxidation. The complex kinetics of reduced XO and XDH with their preferred electron acceptors has been reviewed by Hille and Nishino (1995). Reaction of XO with O_2 is biphasic; a fast two-electron transfer which generates hydrogen peroxide (H_2O_2) and a slower one-electron transfer to produce superoxide anion (O_2^-). XO shows negligible activity with NAD^+. Re-oxidation of XDH via NAD^+ is a monophasic two-electron reaction. Surprisingly, the dehydrogenase form is more efficient than XO at generating O_2^- although NAD^+ is the preferred substrate. For rat liver XDH, $K_m^{Oxygen} = 260\ \mu M$ whereas $K_m^{NAD} = 8.5\ \mu M$ (Saito and Nishino 1989; Nishino 1994). In contrast, AO is without reactivity towards NAD^+ (Li Calzi *et al.* 1995; Turner *et al.* 1995) and lacks an amino sequence characteristic of a NAD^+-binding site, which is conserved in XO proteins (Li Calzi *et al.* 1995; Kurosaki *et al.* 1999; Wright *et al.* 1999a).

Substrate specificity

AO and XO catalyse the oxidation of an extensive range of *N*-heterocycles and aldehydes; of the two enzymes AO has a wider substrate specificity than XO (Beedham 1985, 1987, 1998). This is illustrated in the following section exemplified, where possible, by *in vivo* or clinical data.

OXIDATION

Oxidation of uncharged N-heterocycles

Table 5.1 shows the major groups of uncharged *N*-heterocyclic substrates of AO. A π-deficient ring system containing a *N*-heteroatom is invariably essential for activity

Table 5.1 Uncharged *N*-heterocyclic substrates of AO

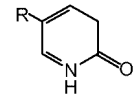
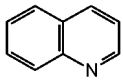
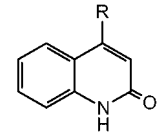
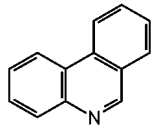
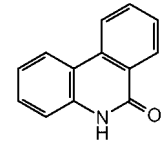
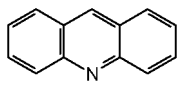
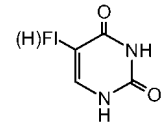
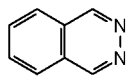
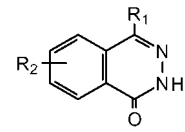
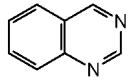
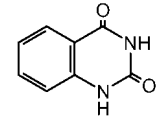
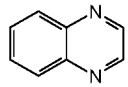
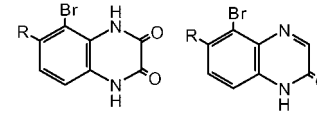
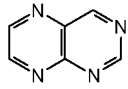
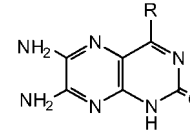
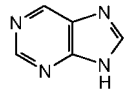
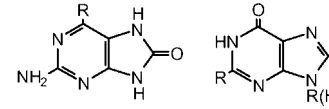
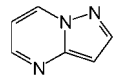
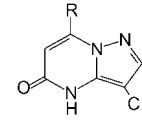
Azaheterocyclic nucleus	Substituted drugs/ xenobiotics	AO-generated metabolite(s)
Pyridine 	Metyrapone AVS	
 Quinoline Isoquinoline	Aminoquinolines Quinine Quinidine	
Phenanthridine 		
Acridine 	*N*[(2′-Dimethylamino) ethyl]acridine-4-carboxamide (DACA)	
Pyrimidine 	2-Pyrimidinones 4-Pyrimidinone IPdR	
Phthalazine 	Substitued phthalazines Carbazeran	

Table 5.1 (*continued*)

Azaheterocyclic nucleus	Substituted drugs/ xenobiotics	AO-generated metabolite(s)
Quinazoline 	Substituted quinazolines	
Quinoxaline 	Substituted quinoxalines Brominidine	
Pteridine 	Methotrexate	
Purine 	Hypoxanthine, Famciclovir 6-Deoxypenciclovir O^6-Benzylguanine 6-Thioguanine 6-mercaptopurine	
Pyrazolopyrimidine 	Zaleplon	

AVS, 2(R,S)-1,2-bis(nicotinamido)propane; IPdR, 5-Iodo-2-pyrimidinone-2′-deoxyribose

towards molybdenum hydroxylases (Beedham 1985, 1987) although unsubstituted pyridine is a very poor substrate for both AO and XO (Krenitsky *et al*. 1972; Stubley and Stell 1980). Of the few pyridine AO substrates, metapyrone is converted to an $\alpha-$ pyridone metabolite by rat liver AO (Usanky and Damani 1983) and AVS, 2(R,S)-1,2-bis(nicotinamido)propane, which is effective in the treatment of vasospasm in dogs following subarachinoid haemorrhage, is metabolised in rabbits to a monopyridone that accounts for ~ 30% total urinary radioactivity (Ishigai *et al*. 1998).

Increased lipophilicity, by fusion of benzene rings to the pyridine nucleus, facilitates binding of substrates to the active site although the position of the hydrophobic groups is critical in governing substrate activity. Quinoline and the isomeric isoquinoline (Table 5.1) give K_m values of 3 mM and 0.2 mM respectively with rabbit liver AO (Stubley *et al*. 1979). 3,4-Benzoquinoline (phenanthridine) has a very high affinity for AO (Stubley and Stell 1980; Rashidi Shagoli 1996), acridine (2,3-benzoquinoline) is a reasonable substrate (McMurtrey and Knight 1984), whereas 5,6- and 7-8-benzoqui-

nolines do not bind to the enzyme (Stubley and Stell 1980). All four cinchona alkaloids, quinine, quinidine, cinchonine and cinchonidine, are oxidised by AO to quinolone metabolites (Beedham *et al*. 1992). These quinoline-based antimalarials have low K_m values with AO but are coupled with relatively low oxidation rates. Consequently, quinine and quinidine are also substrates for cytochrome P450, principally CYP3A (Spray 1996). In healthy volunteers, 3-hydroxyquinine, quinine glucuronide and 2′-quininone are found as major urinary metabolites of quinine (Figure 5.3) (Wanwimolruk *et al*. 1995).

In contrast, the acridine anticancer agent *N*-[(2′-dimethylamino)ethyl]acridine-4-carboxamide, DACA (Figure 5.4), is rapidly converted to an acridone metabolite by human, rat and guinea pig AO (Schofield *et al*. 2000) but is also hydroxylated by cytochrome P450 in rats and mice (Robertson *et al*. 1993). However, neither phenolic nor glucuronide metabolites were detected in urine during Phase I clinical trials whereas all the major metabolites contained an acridone nucleus (Schofield *et al*. 1999). Oxidation of DACA by AO is unusual in that the normal position of oxidation, adjacent to a heterocyclic nitrogen atom, is not available for enzyme attack and oxidation occurs at an alternative electron-deficient site in the molecule (Figure 5.4, Table 5.1).

The diazabenzenes, pyrimidine, pyrazine and pyridazine, show little or no activity with either rabbit liver AO or bovine milk XO (Krenitsky *et al*. 1972; Stubley and Stell 1980). Oxidation of 2-pyrimidinone to uracil (Figure 5.5) is widely used as an

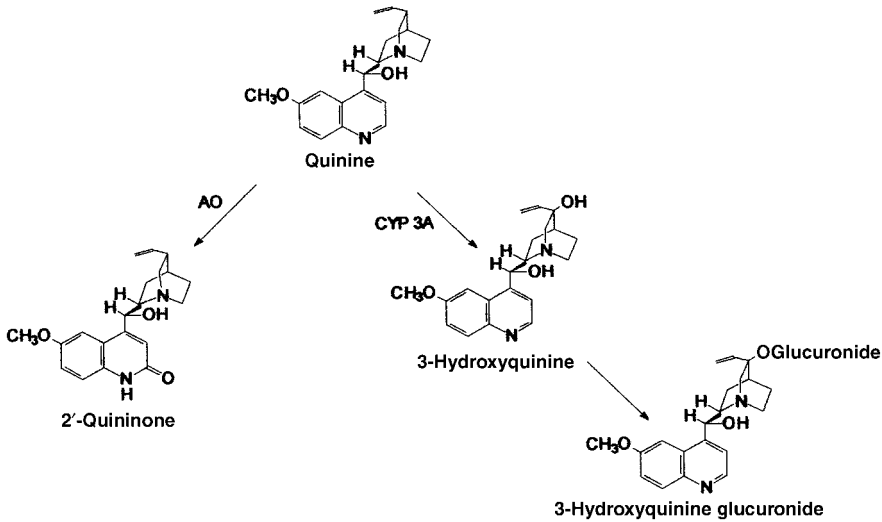


Figure 5.3 Major urinary metabolites of quinine in healthy volunteers (Wanwimolruk *et al*. 1995).

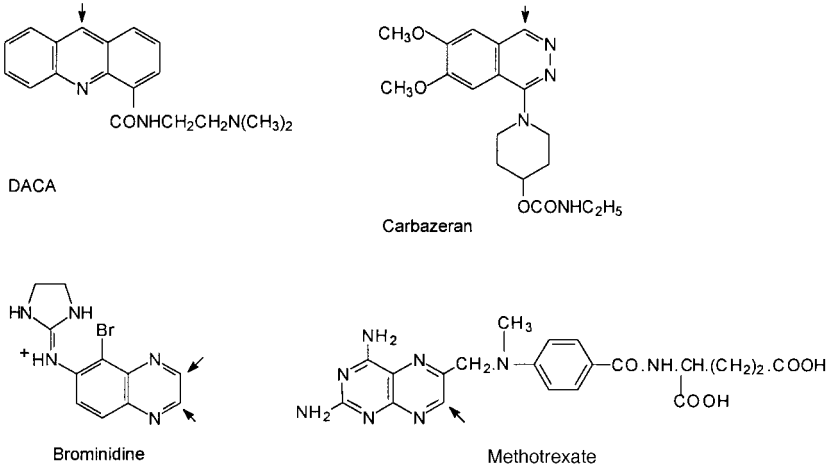


Figure 5.4 *In vivo* substrates of AO.

electron-donating system for AO in *in vitro* reduction studies. Although XO can also catalyse the same reaction, oxidation rates are slow and thus in combined enzyme preparations and liver slices AO is the major 2-pyrimidinone-oxidising enzyme (Oldfield 1998). In fact, 2-pyrimidinone oxidation exhibits biphasic kinetics and it is thought that two AO isozymes are present in guinea pig liver (Yoshihara and Tatsumi 1986; Oldfield 1998). Uracil is also a product of 4-pyrimidinone oxidation which is catalysed by both AO and XO (Oldfield 1998). Results from liver slice incubations show that the latter enzyme predominates in the oxidation reaction (Figure 5.5).

AO-catalysed oxidation of 2-pyrimidinones has been proposed as a bioactivation route for a number of pro-drugs. These include 5-ethynyluracil, a mechanism-based inhibitor of dihydropyrimidine dehydrogenase (Porter *et al.* 1994), 5-fluoro-2-pyrimidinone, a precursor of 5-fluorouracil (Guo *et al.* 1995) and IPdR, which is activated to the radiosensitising nucleoside, 5-iodo-2′-deoxyuridine (Chang *et al.* 1992; Kinsella *et al.* 1998). 5-Fluoro-4-pyrimidinone is also oxidised to 5-fluoruracil but in this case XO

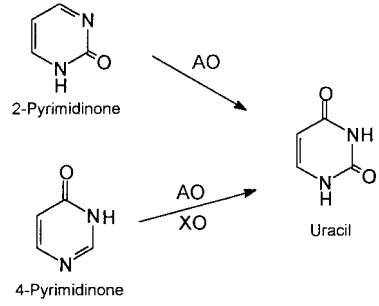


Figure 5.5 Oxidation of 2-pyrimidinone and 4-pyrimidinone to uracil catalysed by AO and XO (Oldfield 1998).

may play the major role (Oldfield 1998). Zaleplon, a pyrazolo[1,5-*a*]pyrimidine derivative (Figure 5.6) recently introduced as an ultra-short acting hypnotic, undergoes *N*-dealkylation and oxidation to *N*-desethylzaleplon and 5-oxo-zaleplon respectively. 5-Oxozaleplon is the major metabolite in human plasma and AO is thought to be responsible for the deactivation step (Kawashima *et al*. 1999).

Phthalazine, quinazoline, quinoxaline and cinnoline ring systems are all oxidised to lactam metabolites by AO (Table 5.1) (Stubley *et al*. 1979; Beedham *et al*. 1990, 1995a). Quinazoline and quinoxaline undergo sequential attack to di-oxo products. Significant interspecies differences in substrate specificity were found although lipophilic substituents facilitated binding of substituted phthalazines and quinazolines to AO. *In vivo* activity towards this group of compounds is exemplified by carbazeran, a 5,6-dimethoxyphthalazine (Figure 5.4), which was developed as an inotropic agent but was found to undergo complete clearance presystemically via 4-oxidation catalysed by liver AO (Kaye *et al*. 1984, 1985). However, AO activity is not restricted to liver (see below). The ocular pharmacokinetics and metabolism of the potent ocular hypotensive drug, brominidine (Figure 5.4), has been studied in rabbits. After rapid ocular absorption, brominidine was metabolised to three metabolites, which were characterised as a quinoxaline-2-3-dione derivative and two isomeric quinoxalinones (Acheampong *et al*. 1995). The same metabolites were also formed in the presence of rabbit and rat liver AO. Diazanaphthalenes show weak or negligible activity towards XO (Krenitsky *et al*. 1972; Stubley *et al*. 1979; Beedham *et al*. 1990).

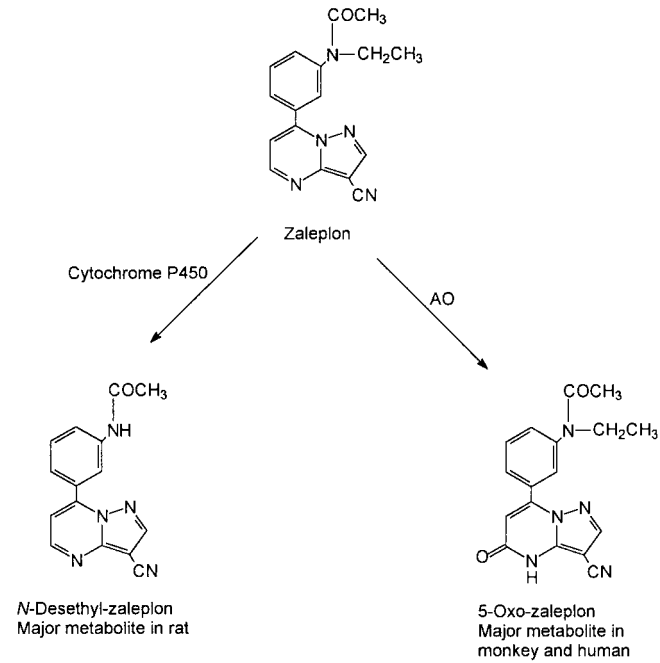


Figure 5.6 Major metabolites of Zaleplon in rat, monkey and human (Chaudhary *et al.* 1994).

Pteridines are excellent substrates for the molybdenum hydroxylases; AO and XO catalyse oxidation at carbon 2,4, or 7 although not necessarily at the same position or in the same molecule (Krenitsky *et al*. 1972; Hodnett *et al*. 1976). In contrast, oxidation has not been reported at carbon 6. As oxidation can occur at alternative positions and substitution effects are complex, prediction of the site of enzymic attack and nature of attacking enzyme is difficult. *In vitro* studies indicate that XO may predominate in pteridine metabolism, however, the antineoplastic drug, methotrexate (MTX, Figure 5.4), is a substrate for AO (Johns *et al*. 1966; Jordan *et al*. 1999) but a competitive inhibitor of XO (Lewis *et al*. 1984). *In vitro* oxidation rates for the conversion of MTX, a 2,4-diaminopteridine, to 7-hydroxymethotrexate are relatively slow, particularly with human liver enzyme (Jordan *et al*. 1999; Kitamura *et al*. 1999c). However, 7-hydroxymethotrexate is the major MTX metabolite; it is also cytotoxic and may contribute to MTX toxicity (Smeland *et al*. 1996). In fact, the term '7-hydroxy'-MTX is misleading as this metabolite is not a phenol but a cyclic lactam and thus it is not conjugated but excreted directly via the kidney. The cyclic lactam structure is typical of all AO- and XO-generated metabolites (Table 5.1).

Krenitsky's group has reported a number of quantitative investigations into the specificity of rabbit liver AO, bovine milk and human liver XO towards substituted purines (Krenitsky *et al*. 1972, 1986; Hall and Krenitsky 1986). Despite these rigorous and extensive studies, it was concluded that there is no single or simple set of determinants that define substrate specificity of purines towards AO and XO. This is mirrored with other substrate groups (Beedham *et al*. 1990, 1995a). With purines and other ring systems, containing more than two N-atoms, there are multiple oxidation sites and the site of attack varies with different substituents. Each enzyme may catalyse oxidation at the same or a different site. This may indicate that there are multiple productive orientations within the substrate-binding site or that the binding site has considerable flexibility.

Purine is sequentially oxidised, via hypoxanthine and xanthine, to uric acid by XO. Indeed the role of XO in endogenous purine metabolism is well documented (Moriwaki *et al*. 1999). The high efficiency of XO towards hypoxanthine and xanthine *in vitro* and *in vivo* indicates that XO activity towards purine-based drugs would be more significant than that of AO. However, AO has the wider substrate specificity and the results obtained with rabbit liver AO may not reflect the reaction of purines with AO from other species. This is illustrated with the antiviral drug, famciclovir, which is the 6-deoxy-diacetyl ester of the herpes active compound penciclovir (Perry and Wagstaff 1995). After oral administration, famciclovir, a pro-drug, is sequentially deacetylated to 6-deoxypenciclovir followed by AO-catalysed oxidation at carbon 6 to penciclovir. Famciclovir and 6-deoxypenciclovir are efficient substrates for AO whereas they show little or no activity with XO (Clarke *et al*. 1995; Rashidi *et al*. 1997). There are two alternative sites for oxidative attack on 6-deoxypenciclovir and famciclovir, carbon 6 and carbon 8 (Figure 5.7). 6-Deoxypenciclovir is oxidised by rabbit liver AO to 8-oxo-6-deoxypenciclovir and penciclovir in approximately equal amounts (Rashidi *et al*. 1997). Both reactions were completely inhibited by the potent AO inhibitor, menadione, and similar K_m values were obtained for each reaction indicating the participation of a single isozyme. In comparison, with guinea pig, human and rat liver AO, the 6-oxidation pathway is predominant with minimal

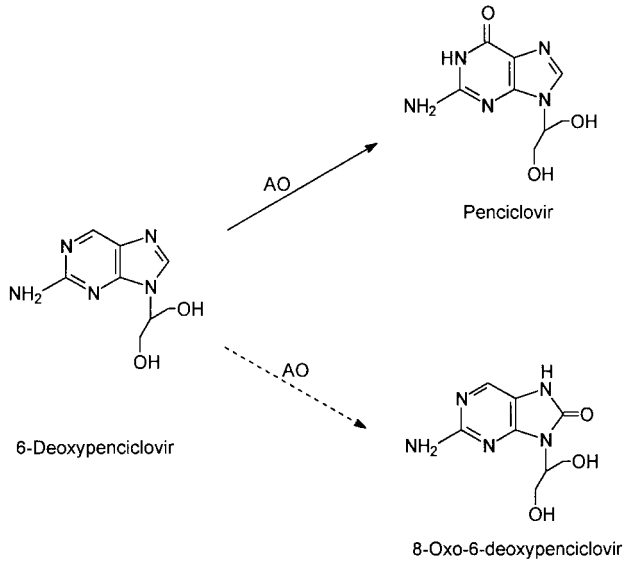
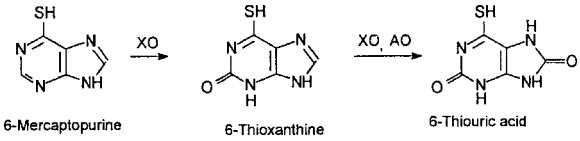


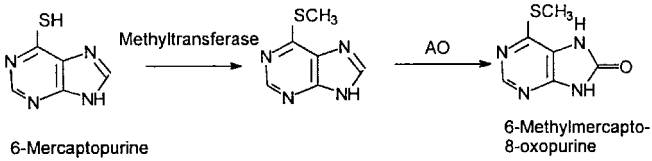
Figure 5.7 AO-catalysed activation of 6-deoxypenciclovir to the antiviral agent, penciclovir, in man and guinea pig (Rashidi *et al*. 1997; Filer *et al*. 1994). (Discontinuous line represents a minor route of metabolism.)

8-hydroxylation. This is consistent with *in vivo* results where penciclovir is the major metabolite accounting for 80-85% dose of famciclovir (Filer *et al*. 1994). AO and XO have also been implicated in the metabolism of other purine-based antivirals such as aciclovir (De Miranda and Good 1992) and 6-deoxycarbovir (Iyer *et al*. 1992).

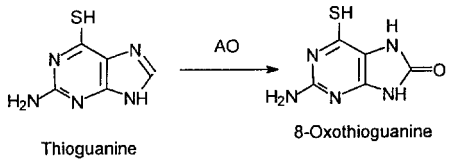
Nevertheless 8-oxidation of xenobiotic purines is observed *in vivo* and, depending on the substrate, may be catalysed by either AO or XO. The major metabolite of oral 6-mercaptopurine is 6-thiouric acid with an alternative catabolic pathway of *S*-methylation (Figure 5.8). Oxidation is probably catalysed by gut XO and co-administration of allopurinol with 6-mercaptopurine increases bioavailability of the latter drug (Zimm *et al*. 1983). 6-Thioxanthine is thought to be the intermediate in the catabolic pathway, thus oxidation at carbon 2 precedes that at carbon 8 (Zimm *et al*. 1984; Rashidi 1996). However during intravenous infusion, which bypasses intestinal XO, of either 6-mercaptopurine or the more potent 6-thioguanine, the major plasma metabolites are 8-oxo-derivatives; 6-methylmercapto-8-oxopurine and 8-oxothioguanine respectively (KeuzenkampJansen *et al*. 1996; Kitchen *et al*. 1999). Although the formation of the 8-oxopurines could be ascribed to either XO or AO, the difference in metabolite profile between oral and intravenous administration indicates that AO may be the significant enzyme in 8-hydroxylation. Similarly, O^6-benzylguanine, an inactivator of O^6-alkylguanine-DNA alkyltransferase, is also oxidised at carbon 8 by human liver AO (Roy *et al*. 1995).



(a) Major metabolic route after oral administration of 6-mercaptopurine



(b) Major metabolic route after intravenous administration of 6-mercaptopurine



(c) Major metabolic route after intravenous administration of 6-thioguanine

Figure 5.8 Oxidative metabolism of 6-mercaptopurine and 6-thioguanine after oral and intravenous administration (Zimm *et al.* 1984; Rashidi 1996; KeuzenkampJansen *et al.* 1996; Kitchen *et al.* 1999).

Oxidation of N-heteroaromatic cations

Quaternisation of a ring nitrogen atom activates a heterocyclic nucleus towards nucleophilic attack. Many heterocyclic cations are, therefore, excellent substrates for AO (Rajagopalan and Handler 1964b; Krenitsky *et al.* 1972; Taylor *et al.* 1984; Beedham *et al.* 1987a) although activity towards XO is only observed at high pH values (Bunting and Gunasekara 1982). Substituent effects and species variation in the oxidation of heterocyclic cations by AO and XO have been reviewed by Beedham (Beedham 1985, 1987).

(a) Oxidation of stable iminium ions

In many substrates, oxidation of N-heteroaromatic iminium ions can occur at two alternative electropositive positions, only one of which is adjacent to the N-hetero-atom (Figure 5.9). N^1-Methylnicotinamide, a niacin catabolite, is simultaneously

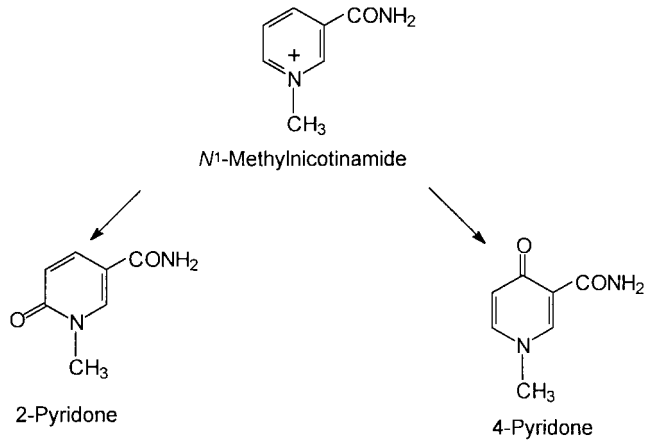


Figure 5.9 Oxidation of N^1-methylnicotinamide to N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-5-carboxamide (Shibata 1989; Egashira *et al.*1999).

converted to N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-5-carboxamide (Shibata 1989; Egashira *et al.*1999) and N-alkyl and N-alkylquinolinium salts are oxidised to isomeric 2- and 4-quinolones by AO (Taylor *et al.* 1984; Beedham *et al.* 1987a). In both cases, the position of oxidation varies with species and substituent (Felsted and Chaykin 1967; Ohkubo *et al.* 1983; Shibata, 1989; Taylor *et al.* 1984; Beedham *et al.* 1987a). However, it is not clear whether both reactions are catalysed by one AO isozyme. Ohkubo *et al.* (1983) separated three molybdenum hydroxylase fractions from rat liver, all of which catalysed the oxidation of N^1-methylnicotinamide; XO, which only produced the 2-pyridone, N^1-methylnicotinamide oxidase I, which preferentially formed the 2-pyridone, and N^1-methylnicotinamide oxidase II, which only formed the 4-pyridone. The two latter isozymes differed in inhibitor sensitivity, heat stability and pH optimum. In contrast, Wright *et al.* (1999a) have recently reported that rat liver AO is most likely expressed as a single gene but that distinct kinetic forms may arise from variations in redox state.

(b) Oxidation of unstable iminium ions

Iminium ions may also be generated as intermediates during the metabolism of cyclic, aliphatic, secondary and tertiary amines such as indoles, prolidines, piperidines and dihydropyridines via cytochrome P450 or monoamine oxidase (Diaz and Squires 2000; Nguyen *et al.*, 1979; Whittlesea and Gorrod 1993; Rodrigues *et al.* 1994; Lin *et al.* 1996; Wu *et al.* 1988; Yoshihara and Ohta 1998). These unstable iminium ions are highly reactive and can react with glutathione and cellular macromolecules (Whittlesea and Gorrod 1993; Skordos *et al.* 1998) or cause neurotoxicity, e.g. 1-methyl-4-phenyl-2,3-dihydropyridinium, $MPDP^+$ (Wu *et al.* 1988; Yoshihara and Ohta 1998; Yoshihara *et al.* 2000). Further oxidation of iminium ions to cyclic lactams by AO (Figure 5.10) represents an important detoxification step for these compounds and

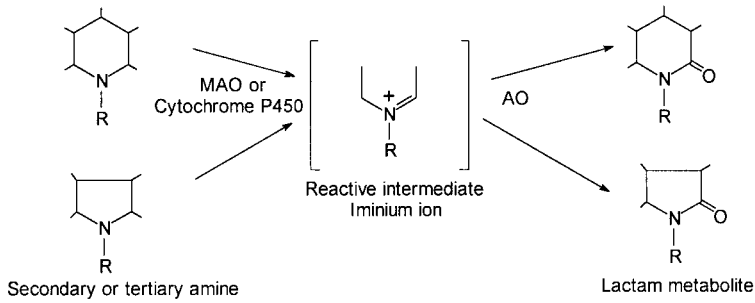


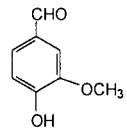
Figure 5.10 Detoxification of reactive iminium ions, generated *in vivo* by cytochrome P450 or MAO, via AO-catalysed oxidation.

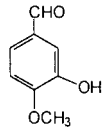
differential activity of AO between tissues and species may be significant. Lactam metabolites from aliphatic amines may represent a minor pathway as in the case of thioridazine (Lin *et al.* 1993, 1996) or the major metabolic route as for nicotine and prolintane (Nakajima *et al.* 1996, 2000; Whittlesea and Gorrod 1993). Cotinine formation may also be important in controlling nicotine levels in the brain (Jacob *et al.* 1997).

Oxidation of aldehydes

AO and XO catalyse oxidation of aldehydes to carboxylic acids. However carboxylic acids are also produced via other enzymes, principally NAD-dependent aldehyde dehydrogenases (ALDH). For example, acetaldehyde is produced from ethanol by alcohol dehydrogenase or CYP2E1; subsequent oxidation to acetic acid can either be catalysed by ALDH, AO or XO. Even though the K_m values of acetaldehyde for the molybdenum hydroxylases are relatively high (36 – 130 mM with XO and 1mM for AO) (Fridovich 1966; Morpeth 1983; Rajagopalan and Handler 1964b), it has been proposed that oxidative injury to liver and pancreas during ethanol metabolism is mediated by the ROS generated from the combined activities of AO and XO (Shaw and Jayatilleke 1992; Wright *et al.* 1999b). Increased ROS production in mammary tissue during alcohol metabolism, via alcohol dehydrogenase and XO, is also thought to lead to DNA damage leading to breast cancer (Wright *et al.* 1999b).

Other aliphatic aldehydes are also substrates for AO and XO although K_m values are higher than corresponding values with ALDH. Studies on the substrate specificity of AO and XO towards aliphatic and aromatic aldehydes up to 1987 has been reviewed previously (Beedham 1985, 1987). Hydrophobicity enhances affinity of aldehydes towards AO and many aromatic aldehydes are excellent substrates including benzaldehyde, indole-3-aldehyde, vanillin, retinal and pyridoxal (Johns 1967; Rashidi 1996; Peet 1995; Panoutsopoulos 1994; Huang *et al.* 1999; Krenitsky *et al.* 1972); lower activities are observed with XO. In a systematic study with 11 structurally related benzaldehydes, Panoutsopoulos (1994) has shown that the presence of a 3-hydroxy group is a critical factor influencing binding and oxidation rates. Vanillin (Figure 5.11) was rapidly oxidised by AO with negligible contribution from XO or





4-Hydroxy-3-methoxy benzaldehyde (vanillin)

$K_m = 15 - 27\ \mu M$

3-Hydroxy-4-methoxy benzaldehyde (isovanillin)

$K_I = 4\ \mu M$

Figure 5.11 Differential reactivity of isomeric hydroxy-benzaldehydes towards AO (Panoutsopoulos 1994; Rashidi 1996).

ALDH whereas the isomeric aldehyde, isovanillin, was predominantly transformed by ALDH but was a potent AO inhibitor (Table 5.2). In addition, AO and ALDH both catalyse oxidation of aldehydes derived from phenylethylamines with little contribution from XO. Although it has been proposed that human liver ALDHs are exclusively responsible for the metabolism of biogenic aldehydes and neurotransmitters (Pietruszko *et al*. 1991), we have shown that AO plays a major role in homovanillamine and 5-hydroxylindoleacetaldehyde metabolism in guinea pig liver and brain (Beedham *et al*. 1995b; Peet 1995; Laljee 1998).

With respect to drug metabolism, acid metabolites resulting from oxidation of dimethylamino groups in the nonsteroidal antioestrogen, tamoxifen, and the antidepressant, citalopram (Figure 5.12), are thought to arise from the combined action of either cytochrome P450 or monamine oxidase and AO (Ruenitz and Bai, 1995; Rochat *et al*. 1998).

REDUCTION

In vitro reduction, catalysed by AO or XO, can be demonstrated under hypoxic or anaerobic conditions in the presence of an appropriate electron donor such as 2-pyrimidinone (AO), benzaldehyde (AO) or xanthine (XO). However, extrapolation of *in vitro* results to *in vivo* metabolism is often difficult to assess for the following reasons: (a) many reduction reactions are only observed under strictly anaerobic conditions or are inhibited by high oxygen concentrations, (b) reduction rates are dependent on the availability and concentration of electron donor, (c) there is overlapping substrate specificity between the molybdenum hydroxylases and other reducing enzyme systems such as cytochrome P450 reductase and NADPH-benzoquinone reductase (DT-diaphorase), (d) reduction may also occur in the intestine due to the action of the gut bacteria, and (e) reduction products may not be detected *in vivo* due to re-oxidation back to the parent compound via microsomal cytochrome P450 or flavin monooxygenase.

Nevertheless, AO has a broad substrate specificity and is less sensitive towards oxygen inhibition than XO (Wolpert *et al*. 1973). In addition, AO may also function as the terminal reductase in coupled electron transfer systems with XO or NADPH-

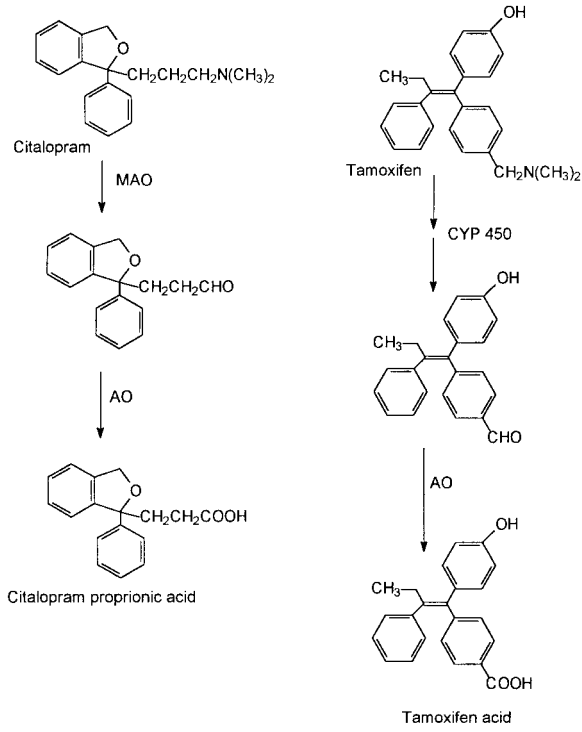


Figure 5.12 The role of AO in the formation of acid metabolites from Citalopram and Tamoxifen.

cytochrome c reductase (Kitamura and Tatsumi 1983a; Tatsumi *et al*. 1982; Kitamura *et al*. 1981). Thus it is likely that, in addition to oxidation reactions, AO-catalysed reduction reactions may be significant *in vivo*. Although there are many reports of AO-catalysed reduction reactions, principally arising from the laboratories of Kitamura, Sugihara and Tatsumi, this area has not been reviewed. Consequently, the major classes of oxidising substrates for AO and XO are described in the following section.

Hepatic AO catalyses the reduction of many *N*-functional groups including nitro groups, *N*-oxides, oximes, azo dyes, *N*-nitroso and *N*-hydroxycarbamoyl substituents. XO shows weak activity as a nitro- and *N*-oxide reductase but may be important in nitrate reduction and subsequent production of nitric oxide (NO).

Nitroreduction

An early investigation by Westerfield *et al*. (1957) described the effect of tungstate administration on the *in vivo* reduction of 4-nitrobenzene sulphonamide in rats. Nitroreduction to sulphanilamide was decreased in tungsten-treated rats compared to control animals and minimal XO activity was detected in tungsten-treated animals. However, 4-nitrobenzensulphonamide was only a weak substrate for XO and it seems

more likely that AO was the molybdenum hydroxylase responsible for the reduction in this case. Indeed, nitrofurazone and the carcinogenic 4-nitroquinoline-N-oxide were reduced to the hydroxylamines by AO with highest nitroreductase activity in rabbit liver and lower activity in rat and guinea pig (Wolpert *et al*. 1973). The relative contribution of the combined molybdenum hydroxylases towards nitrofurazone reduction compared to microsomes was around one third total activity in rat, mouse, hamster and rabbit and around one fifth in guinea pig liver. Nitrated polycyclic hydrocarbons are reduced by bovine milk XO (Bauer and Howard 1990) and rabbit liver AO (Tatsumi *et al*. 1986) to hydroxylamines which may subsequently bind to DNA.

Reduction of nitrates by XO

Reduction of organic nitrates and inorganic nitrate or nitrite to nitric oxide (NO), supported by either xanthine or NADH, is catalysed by XO under anaerobic conditions (Millar *et al*. 1998; Doel *et al*. 2000; Godber *et al*. 2000). It is proposed that XO may be important in the metabolism of organic nitrates such as glyceryl, isoamyl and isobutyl nitrate to NO. Furthermore, the observed inactivation of XO by NO may serve to explain the phenomenon of tolerance with these compounds. Generation of NO from XO is dependent on low O_2 tension and inhibited by oxipurinol. Millar *et al*. (1998) suggested that XO may act as a source of NO derived from endogenous nitrate and nitrite under ischaemic conditions when NO synthase does not function.

N-oxide reduction

XO will also function as an *N*-oxide reductase under anaerobic conditions; thus 3-amino-1,2,4-benzotriazine-1,4-dioxide is reduced by bovine milk XO to the mono-*N*-oxide (Laderoute and Rauth 1986). However, nicotinamide-*N*-oxide, imipramine-*N*-oxide, cyclobenzaprine-*N*-oxide and *S*-(−)-nicotine-1′-*N*-oxide are reduced to their parent amines by rat and rabbit liver AO (Kitamura and Tatsumi 1984a,b; Sugihara *et al*. 1996b). XO also catalyses *S*-(−)-nicotine-1′-*N*-oxide reduction to nicotine *in vitro* although AO is much more efficient in this respect (Sugihara *et al*. 1996b). Interconversion of *S*-(−)-nicotine-1′-*N*-oxide to nicotine *in vivo* may serve to prolong nicotine action via a futile cycle of oxidation-reduction reactions. Nicotine-1′-oxide and cotinine are the major *in vivo* metabolites of *S*-(−)-nicotine-1′-*N*-oxide in humans (Berkman *et al*. 1995).

Oxime reduction

Acetophenone oxime, salicylaldoxime and benzamidoxime are reduced by cytosolic AO under anaerobic conditions whereas no reaction was observed in the presence of electron donors of XO, DT-diaphorase or rabbit liver microsomes (Tatsumi and Ishigai 1987). Acetophenone oxime and salicylaldoxime were converted to the corresponding oxo compounds and benzamidoxime to a ketimine (Figure 5.13). *In vivo* studies in rats indicate that butanal oxime, an anti-skinning agent in varnishes and paints, is reduced to an imine via AO, which undergoes subsequent hydrolysis and conversion

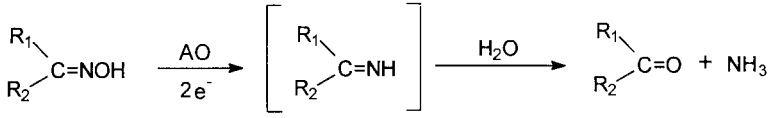


Figure 5.13 Proposed mechanism for oxime reduction catalysed by AO (Tatsumi and Ishigai 1987).

of butanal to carbon dioxide. The reductive reaction may protect against butanal oxime toxicity via cyanide production in an alternative metabolic pathway catalysed by cytochrome P450 (Mathews *et al*. 1998).

Reduction of azo dyes

Azo dyes are widely used as colourants in drugs, food and beverages (Wolff and Oehme 1974). Detoxification of these potentially carcinogenic compounds is usually associated with reduction of the azo link to a primary amine. However, reductive metabolism of some azo compounds may produce carcinogenic or mutagenic products (Chung 1983). Rabbit liver AO has been shown to possess significant azoreductase activity towards methyl red, methyl orange and other dimethyl aminoazobenzenes (Kitamura and Tatsumi 1983b; Stoddart and Levine 1992). Lipophilic azo dyes, which are readily reduced by microsomal cytochrome P450, were weak substrates whereas water soluble or charged azo dyes were readily reduced by the cytosolic enzyme (Stoddart and Levine 1992).

Reduction of N-functional groups

Rabbit and guinea pig liver AO also catalyse the reduction of aromatic and heterocyclic hydroxamic acids to amides (Sugihara and Tatsumi 1986), nitrosoamines to hydrazines (Tatsumi and Yamada 1982; Tatsumi *et al*. 1983), and *N*-hydroxyurethane to urethane (Sugihara *et al*. 1983) in the presence of an electron donor.

Sulphoxide reduction

AO acts as a sulphoxide reductase under anaerobic conditions e.g. sulindac, sulphinpyrazone, phenothiazine sulphoxide, dibenzyl sulphoxide and diphenylsulphoxide (Lee and Renwick 1995; Yoshihara and Tatsumi 1986, 1990; Tatsumi *et al*. 1982). Reduction of sulindac and sulphinpyrazone produces active sulphide metabolites (Pay *et al*. 1980; Duggan 1981). Lee and Renwick (1995) showed that AO is the main sulindac-reductase in rat and rabbit liver whereas sulindac reduction in kidney cytosol was catalysed either by thioredoxin reductase or a coupled electron transfer system. Diphenyl sulphoxide (DPSO) is reduced to diphenyl sulphide under anaerobic conditions but acts as an inhibitor in the presence of electron acceptors such as molecular O_2 or potassium ferricyanide (Yoshihara and Tatsumi 1986). Although sulphoxides are reduced under hypoxic conditions, re-oxidation of a sulphide to sulphoxide and sulphone metabolites, by microsomal monooxygenases, may occur under normoxia.

Tissue oxygen levels will thus be an important determinant of the overall metabolic fate of a compound. In further studies with DPSO in perfused guinea pig liver, the sulphoxide was exclusively converted to diphenylsulphone under normoxia whereas under hypoxia diphenylsulphone formation decreased in parallel with reducing oxygen concentrations (Yoshihara and Tatsumi 1990). Under hypoxic conditions, the reduction pathway to diphenyl sulphide was only significant in the presence of an AO-reducing substrate such as 2-hydroxypyrimidine or benzaldehyde. Preliminary investigations in rabbit showed that oxidation to diphenyl sulphone and reduction to diphenyl sulphide both occurred after oral administration of diphenylsulphoxide. Chiral inversion of flosequinan (FSO), a peripheral vasodilator, *in vivo* is thought to occur via the formation of flosequinan sulphide (Figure 5.14). Rat liver AO exhibits stereoselective reduction of FSO, reducing R-FSO at a rate ~18 times higher than the S-FSO (Kashiyama *et al*. 1999).

Unlike nitroreduction, XO will not support sulphoxide reduction (Yoshihara and Tatsumi 1986, 1990; Kitamura *et al*. 1999d), although it will transfer electrons from an electron donor such as xanthine to AO in a coupled electron transfer system (Kitamura *et al*. 1981; Kitamura and Tatsumi 1983a).

Reductive dehalogenation

From the above studies it can be seen that XO catalyses a narrower range of reductive reactions than AO, and many of the reactions have much lower reduction rates. A more unusual substrate of XO is 6-bromomethyl-(9H)-purine which undergoes reductive dehalogenation to 6-methylpurine. Not only did XO catalyse the oxidation of 6-bromomethyl-(9H)-purine to its uric acid analogue but also reduced the substrate with concomitant enzyme inhibition by modification of the flavin moiety (Porter 1990).

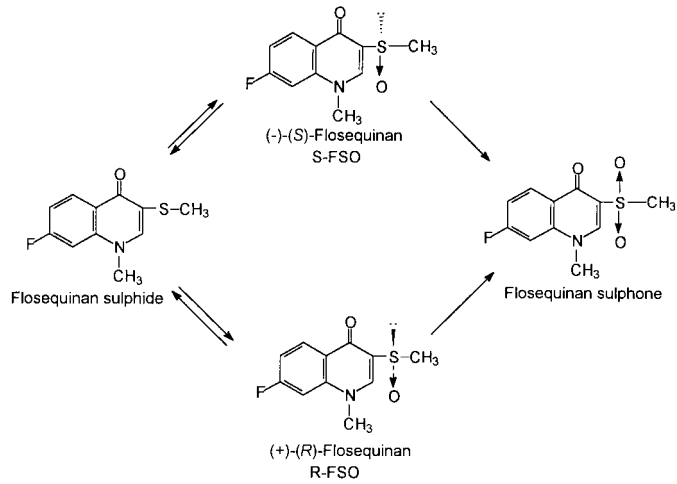


Figure 5.14 In vivo metabolism of flosequinan in the rat (Kashiyama *et al*. 1999).

Reduction in vivo

Reduction can be a significant pathway *in vivo*. Zonisamide, a novel anticonvulsant, is primarily metabolised via a reductive pathway during which the benzisoxazole ring is cleaved to 2-sulphamoylectylphenol (Stiff *et al.* 1990). The mechanism, shown in Figure 5.15, is thought to be analogous to oxime reduction involving formation of the intermediate ketamine and subsequent hydrolysis to the final oxo compound with stoichiometric formation of ammonia (Sugihara *et al.* 1996a). Although the reduction reaction can be catalysed by CYP3A (Nakasa *et al.*1993), cytosolic zonisamide reducing activity in rat, rabbit, guinea pig, mice and hamster liver is higher than microsomal activity (Sugihara *et al.* 1996a). It should also be noted that the same group have shown that this reaction can also be catalysed by gut bacteria (Kitamura *et al.* 1996).

Not surpisingly, Yoshihara and Tatsumi (1986) suggest that AO is one of the major sulphoxide reductases in mammals. Based on current evidence this is not an unreasonable conclusion. AO is an effective reductase and does not require a strict anaerobic environment, unlike XO and cytochrome P450 reductase. Localisation within the liver is in the pericentral zone, an area of low oxygen tension. Furthermore, studies with zonisamide indicate that reduction may be significant *in vivo* pathway. However, AO-catalysed reduction may not be restricted to mammals. Kitamura *et al.* (1999a) has shown that Fenthion sulphoxide is reduced to the organophosphorus pesticide, Fenthion, by AO in the hepatopancreas of goldfish, *Carassius auratus*.

Interaction with inhibitors

Inhibitory activity towards the molybdenum hydroxylases appears to mimic that of substrates. There are common inhibitors such as cyanide, arsenite and methanol, which react with the molybdenum cofactor of both enzymes (Rajagopalan and Handler 1964a; Turner *et al.* 1995; Massey and Harris 1997). Many XO inhibitors are substrate analogues based on the structure of allopurinol, a clinically used XO inhibitor. In contrast, a diverse group of inhibitors, including several drugs, are potent *in vitro* inhibitors of AO. However, there are no reported clinical reactions for any of

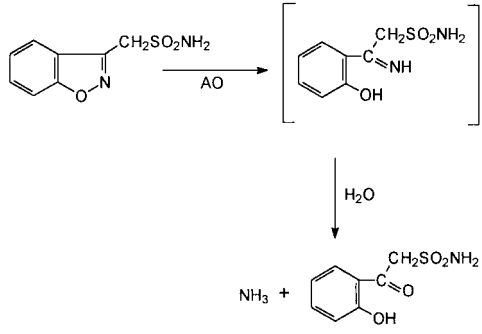


Figure 5.15 Reductive cleavage of Zonisamide catalysed by AO (Sugihara *et al.* 1996).

these compounds. Furthermore, as with AO substrates there is a species variation in the reactivity of AO inhibitors.

INHIBITORS OF AO

Menadione is one of the most potent AO inhibitors (Rajagopalan *et al*. 1962; Rajagopalan and Handler 1964a); it is universally used *in vitro* to characterise enzyme activity and is equipotent with oxidation or reduction reactions and with AO from different species (Yoshihara and Tatsumi 1986; Rashidi *et al*. 1997; Schofield *et al*. 2000). Interaction is thought to occur at the FAD site (Rajagopalan *et al*. 1962; Rajagopalan and Handler 1964a; Yoshihara and Tatsumi 1986), which is consistent with the ability of menadione to act as an electron acceptor of XO (Mahler *et al*. 1955). Consequently, XO-catalysed oxidation rates may be enhanced in the presence of menadione (Rajagopalan *et al*. 1962; Yoshihara and Tatsumi 1986) and ambiguous results obtained with substrates of both molybdenum hydroxylases in cytosol, hepatocytes, liver slices and partially purified enzyme fractions. This is illustrated by the oxidation of 4-pyriminidone to uracil (Figure 5.5), catalysed by AO and XO, in guinea pig liver, which is inhibited by 10 μM but not 100 μM menadione (Oldfield 1998). Menadione inhibition has not been studied *in vivo* but it is unlikely to reduce AO activity because it is rapidly reduced by DT-diaphorase (Thor *et al*. 1982) or even XO (Mahler *et al*. 1955).

Table 5.2 shows the most potent AO inhibitors identified *in vitro,* the majority of which have reported kinetic constants (IC_{50} or K_i values) of the order of 1 μM for either rabbit, rat, guinea pig or human liver AO. In most cases, kinetic constants have been measured against substrate oxidation but, where tested, similar results have been obtained against reduction.

Although the potency of inhibitors such as menadione, β-oestradiol, chorpromazine and amsacrine is similar with animal and human liver, other compounds do not give consistent results. In particular methadone (Robertson and Gamage 1994) and proadifen (SKF 525A) (Robertson and Bland 1993), which were originally identified as potent inhibitors of rat liver enzyme, show little reaction with human or guinea pig hepatic AO (Schofield *et al*. 2000). In contrast, Rashidi *et al*. (1995) found a close correlation ($r = 0.96$) between inhibitor reactivity towards guinea pig and human liver AO for 13 drugs with varying inhibitory potency.

INHIBITORS OF XO

Numerous compounds have been tested as XO inhibitors (Hille and Massey 1981); nevertheless there is less structural diversity among XO inhibitors than those of AO as most effective XO inhibitors are purine–based analogues. Structure-activity relationships for XO-inhibitors have been reviewed by Beedham (1987). The prototype inhibitor, allopurinol, is a potent mechanism-based inhibitor of XO (Massey *et al*. 1970) but has weak inhibitory activity towards AO (Hall and Krenitsky 1986; Rashidi *et al*. 1995; Rashidi 1996). Like menadione, it is extensively used to distinguish between AO and XO activity (Roy *et al*. 1995; Clarke *et al*. 1995; Shanmuganathan *et al*. 1994; Oldfield 1998) although results should be interpreted with caution as

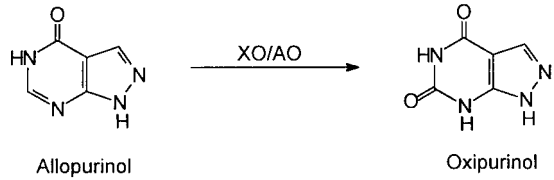
Table 5.2 *In vitro* inhibitors of AO

Inhibitor	Reactivity[a] with			
	Rabbit AO	Rat AO	Guinea Pig AO	Human AO
Menadione	++++	++++	++++	++++
Amsacrine	+++	+++	+++	+++
β-Oestradiol	+++	ND	+++	++++
7-Hydroxy-DACA	ND	++++	++++	++++
Chlorpromazine	ND	ND	++++	++++
Promethazine	ND	ND	+++	+++
Phenothiazine	ND	ND	++++	ND
Hydralazine	+++	ND	++++	+++
Isovanillin	ND	ND	+++	ND
Protocatechuic aldehyde	ND	ND	+++	ND
Dopamine	ND	ND	+++	ND
Quinacrine	+++	ND	ND	ND
Antimycin A	+++	ND	ND	ND
Methadone	ND	++++	+	+
SKF-525A	ND	+++	+	+
Norharman	ND	ND	+++	ND
Benzoquinone	+++	ND	ND	ND
Progesterone	++	ND	ND	ND
Triton X-100	++	ND	ND	ND
Cimetidine	ND	ND	+	++
Disulfiram	ND	ND	++	ND
D-Propoxyphene	ND	++	ND	ND
Amytal	+	ND	ND	ND

[a] Kinetic constants (IC_{50} or K_i value)
+++, < 1 μM; ++, $1 - 10$ μM; ++, $10 - 100$ μM; +, > 100 μM
ND: Not determined
7-Hydroxy-DACA, 7-Hydroxy-*N*[(2′-dimethylamino)ethyl]acridin-6-caboxamide
SKF-525A, Proadifen
(Rajagopalan *et al*. 1962; Rajagopalan and Handler 1964a; Johns 1967; Johnson *et al*. 1985; Yoshihara and Tatsumi 1986; Lee and Chan 1988; Panoutsopoulos 1994; Rashidi 1996; Rashidi *et al*. 1995; Laljee 1998; Schofield *et al*. 2000)

allopurinol is oxidised by both enzymes to oxipurinol (Figure 5.16) (Krenitsky *et al*. 1986; Moriwaki *et al*. 1993a; Yamamoto *et al*. 1993). With XO, oxipurinol is strongly bound to the reduced molybdenum cofactor preventing further electron transfer (Massey *et al*. 1970; Spector *et al*. 1986) whereas oxipurinol does not significantly inhibit AO (Hall and Krentisky 1986, Moriwaki *et al*. 1993a).

Inhibition of XO *in vivo* accounts for the hypouricaemic effect of allopurinol in the management of gout (Star and Hochberg 1993). Although the conversion of allopurinol to oxipurinol has been attributed to XO (Murrell and Rapeport 1986), it is more likely that AO is principally responsible for oxipurinol formation (Reiter *et al*. 1990; Moriwaki *et al*. 1993a). In children administered increasing allopurinol doses, Sweetman (1968) found that maximum conversion of allopurinol to oxipurinol coincided with almost complete inhibition of XO activity. In addition to its clinical use in gout, allopurinol is also effective in augmenting the therapeutic effect of drugs metabolised by XO. Co-administration of allopurinol with oral 6-mercaptopurine increases plasma 6-mercaptopurine levels five fold and also enhances drug toxicity (Zimm *et al*. 1983).



Allopurinol

Oxipurinol

	XO	AO
K_m (μM)	2	380

	XO	AO
K_i (μM)	0.08	150

Figure 5.16 Molybdenum hydroxylase-catalysed oxidation of allopurinol to oxipurinol (Hall and Krenitsky 1986; Krenitsky *et al* 1986).

Allopurinol also has a beneficial effect in immunosuppressive therapy with azathioprine that may be related to XO-inhibition (Chocair *et al.* 1993). In contrast, there are no reported drug interaction between allopurinol and AO substrates such as famciclovir or thioguanine (Fowles *et al.* 1994; Hande and Garrow 1996).

BOF-4242, a pyrazolo[1,5-a]triazine (Figure 5.17), is a recently introduced potent XO inhibitor (K_i for (−)-isomer = 1.2 nM) which has a longer duration of action *in vivo* than allopurinol (Okamoto and Nishino 1995).

Tissue distribution

Tissue distribution of AO and XO has been compared previously in reviews by Beedham (1985, 1987) and Moriwaki *et al.* (1997,1999). Most of the studies described in the two former reviews are based on substrate specific assays in tissue homogenates or purified enzyme, whereas the later review by Moriwaki *et al.* (1997) includes histochemical and immunohistochemical techniques to localise enzyme activity. Limited results were available for gene expression in different species and tissues. The present chapter will concentrate on recent studies carried out on the tissue distribution in common laboratory species and humans. Tables 5.3 and 5.4 compare the tissue distribution of XO and AO respectively, using different assay methods.

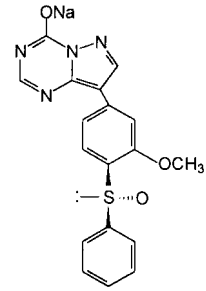


Figure 5.17 (-) Isomer of BOF-4242, pyrazolo[1,5-a]triazine, a tight binding inhibitor of XO (Okamoto and Nishino 1995).

Table 5.3 Tissue distribution of xanthine oxidase

Tissue	Rat	Rabbit	Mouse	Guinea pig	Bovine	Human
Liver	✓✓ (H,I)		✓✓ (E)	✓ (K)	✓ (K,I)	✓ (K,E)
Pericentral	✓✓ (H,I)				✓ (I)	✓ (I,H)
Periportal	✓ (I)					✓✓ (H)
Kupffer Cells					✓ (I)	✓ (I)
Endothelial cells					✓ (I)	
Kidney	✓ (H,I)		✓ (E)	✓ (K)		✓ (K)
Endothelial cells						✓ (I)
Glomerulus Tubules	✓ (H,I)					
Heart	✓ (I)	✓ (K)	✓ (E)		✓ (K)	– (I) ✓ (K)
Adrenals	✓ (H,I)					✓ K
Spleen	✓ (H,I)			✓ (K)		✓ K
Skeletal Muscle	✓ (I)	✓ (K)			✓ (K)	✓ (I,K)
Tongue (epithelial)	✓✓ (H,I)					
Oesophagus	✓✓ (H,I)					
Stomach	✓ (H,I)					
Small Intestine	✓✓✓ (H,I)		✓✓ (E)	✓ (K)		✓✓ (I,K,E)
Large Intestine	✓ (H,I)		✓ (E)	✓ (K)		
Lung	✓✓ (H,I)		✓ (E)	✓ (K)		– (I,K)
Bronchioles	✓✓ (H,I)					
Alveolus	✓✓ (H,I)					
Brain		✓ (K)				– (I) ✓ (K)
Mammary gland			✓ (E)		✓ (I)	✓✓ (I)
Milk					✓✓	✓✓ (I)

✓ XO detected with kinetic measurement (K), Histochemical (H), Immunocytochemistry (I) or mRNA expression (E).
– No activity found.
(Krenitsky *et al.* 1974; Beedham *et al.* 1987b; Wagner and Harkness 1989; Kooji *et al.* 1992; Terao *et al.* 1992, 1997; Wright *et al.* 1993; Xu *et al.* 1994; Moriwaki *et al.* 1993b, 1998a,b; Linder *et al.* 1999; Moriwaki *et al.* 1996).

Enzyme localisation within a tissue may depend on the limitations of a visualisation technique or differences in mRNA expression, transcription or translational events. Thus the comparative tissue distribution can vary with different assay methods. Kinetic measurements (designated K in Tables 5.3 and 5.4) based on specific substrate turnover with a spectroscopic or fluorogenic end-point will give an accurate estimation of functional enzyme within a tissue but may lack sensitivity and give little information on cell-specific localisation. Furthermore, purification of the protein may lead to inactivation of the holoenzyme. While histochemical techniques (H), using enzyme-specific substrates, will also detect functional enzyme, they will differentiate between enzyme activity in different cell sub-types. However, immunohistochemical methods (I) are usually more sensitive to tissues containing lower enzyme activity but there is a risk of cross-reaction with other proteins. Consequently, there is little variation between results obtained from tissues with high molybdenum hydroxylase activity with any of the above techniques (K, H or I). Determination of mRNA expression (E) can give misleading results regarding functional molybdenum hydroxylase activity, as steady-state levels of mRNA do not necessarily correlate with

Table 5.4 Tissue distribution of aldehyde oxidase

Tissue	Rat	Rabbit	Mouse	Guinea Pig	Bovine	Human
Liver	✓✓ (H,I)	✓✓✓ (E,K)	✓ (E,I,K)	✓✓ (K)	✓✓ (I,K,E)	✓ (E)
Pericentral	✓✓ (H,I)			✓✓ (K)		✓ (I)
Periportal	✓ (I)					
Kidney		✓✓ (E,K)		✓ (K)	✓ (I,E)	✓ (E)
Glomerulus Tubules	✓ (H,I)					
Heart	✓ (I)	✓ (E)	✓ (E)			
Spleen			✓ (E,I,K)	✓ (K)	✓ (I,K,E)	
Skeletal Muscle		–				
Tongue (epithelial)	✓ (H,I)					
Oesophagus	✓✓ (I)		✓ (E)		✓ (I)	
Stomach	✓ (H,I)	Trace				
Small Intestine	✓ (I)			✓ (K)		
Large Intestine	✓ (I)			✓ (K)	✓ (I)	
Lung	✓✓ (H,I)	✓✓ (E,K)	✓ (E,I,K)	✓ (K)	✓ (I,K,E)	✓ (E)
Bronchioles	✓✓ (H,I)					
Alveolus	✓ (H,I)					
Brain		✓ (E)	✓✓ (E,I,K)			✓✓ (E)

✓ AO detected with kinetic measurement (K), Histochemical (H), Immunocytochemistry (I) or mRNA expression (E).
– No activity found.
(Krenitsky *et al.* 1974; Wright *et al.* 1995, 1997; Beedham *et al.* 1987b; Li Calzi *et al.* 1995; Bendotti *et al.* 1997; Moriwaki *et al.* 1998a,b; Huang *et al.* 1999; Kurosaki *et al.* 1999)

catalytically active protein (Kurosaki *et al.* 1995, 1999; Li Calzi *et al.* 1995; Linder *et al.* 1999).

Despite the different methods employed, there is a general consensus about relative tissue distribution for each enzyme among all species studied. However, there is some interspecies variation in the expression of XO; thus rabbit tissues show lower XO activity than other species (Wagner and Harkness 1989). In contrast, the specificity of AO varies significantly among species. There are also major differences in tissue distribution between AO and XO (Tables 5.3 and 5.4). Therefore, in humans and mammals, highest XO expression and activities are found in proximal intestine, lactating mammary gland and liver, whereas high AO levels are consistently found in the liver, lung, kidney and brain. Much lower AO activity is present in intestine with no AO mRNA expression in the human mammary gland (Wright *et al.* 1997).

MOLYBDENUM HYDROXYLASE ACTIVITY IN RAT

In rats, XO activity was high in surface epithelium of small intestine, strong to moderate in liver cytoplasm, moderate in surface epithelium of tongue, oesophagus, stomach, bronchioles, alveoli, renal tubules and large intestine but not detected in heart or muscle fibres (Moriwaki *et al.* 1998a). Using enzyme histochemistry, Moriwaki *et al.* (1998a) observed high AO activity in Wistar rat liver with lower activity in surface epithelium of tongue, bronchioles and renal tubules. AO activity in heart, skeletal muscle, stomach, oesophagus small and large intestine were only detected using a more sensitive immunohistochemical technique. Both AO and XO were

localised in the pericentral rather than the periportal zone of the liver (Moriwaki *et al.* 1998a,b). Localisation of the enzymes in this area of low oxygen tension may indicate a role for these enzymes in reduction reactions *in vivo*. However, AO distribution in rat liver is strain/animal dependent (see below).

MOLYBDENUM HYDROXYLASE ACTIVITY IN MOUSE

Although moderate XO activity has been found in mouse intestine and liver, expression of XO mRNA is low in all mouse tissues studied (Terao *et al.* 1992). However, after induction with interferon, Terao *et al.* (1992) showed a rapid elevation of mRNA in mouse liver, kidney, small and large intestine, heart, lung but not in spleen and brain. In contrast, the transcipt coding for mouse AO is expressed at highest concentrations in the oesophagus and in liver, lung, heart, testis with lower levels in brain, spinal cord and eye (Kurosaki *et al.* 1999). AO mRNA was not detected in stomach, skin, striated muscle or small and large intestine using Northern blot analysis. Kurosaki *et al.* (1999) did not find a strict correlation between mRNA levels and functional AO protein; thus there was no detectable AO activity in oesophagus whereas brain AO activity was much higher than would be predicted from mRNA accumulation. It is likely that the synthesis of mouse XO and AO is under translational and post-translational control. Within the CNS, Bendotti *et al.* (1997) have shown that there is specific and high expression of the AO gene in mouse choroid plexus. In addition, AO mRNA is localised in the large motor neurones of the nuclei of facial, motor trigemini and hypoglossus nerves and motor neurones of the anterior horns of the spinal cord.

MOLYBDENUM HYDROXYLASE ACTIVITY IN RABBIT

Rabbit liver and intestine exhibits lower oxidative activity towards xanthine than rat, mice or guinea pig (Krenitsky *et al.* 1974) but xanthine dehydrogenase activity has been demonstrated in heart, skeletal muscle and brain (Wagner and Harkness 1989). Using cDNA cloning, Huang *et al.* (1999) have shown that rabbit retinal oxidase is identical to AO and that retinal oxidase mRNA is widely expressed in tissues; mRNA expression was shown to be very high in liver and lung, relatively high in kidney, pancreas, brain stem and spinal cord with lower expression in stomach and muscle.

MOLYBDENUM HYDROXYLASE ACTIVITY IN GUINEA PIG

Quantitative measurement of AO and XO activity in guinea pig tissues has been compared using phenathridine (AO), phthalazine (AO) and xanthine (XO) as specific enzyme substrates (Beedham *et al.* 1987a). Kidney AO showed approximately 40% of liver activity with lower rates obtained for spleen, intestine and lung. XO activity was highest in jejunum, followed by duodenum, ileum, liver, spleen, kidney and lung. Both molybdenum hydroxylases are located in guinea pig liver cytosol and mitochondria (Critchley *et al.* 1992). Substrate/inhibitor studies have identified AO in guinea pig cortex and striatum (Beedham *et al.* 1995b; Laljee 1998).

MOLYBDENUM HYDROXYLASE ACTIVITY IN BOVINE TISSUE

AO distribution is similar in bovine tissues with high expression of mRNA and functional protein in liver, lung, spleen and brain (Li Calzi *et al.* 1995). Positive immunoreactivity was also observed in kidney, eye, thymus, testis, duodenum, heart and oesophagus although not in striatal muscle and pancreas.

HUMAN MOLYBDENUM HYDROXYLASE ACTIVITY

In contrast to rat liver XO, intense staining of XO protein was found in the cytoplasm of human periportal hepatocytes with little protein expression in pericentral hepatocytes. High XO activity and protein levels were also seen in the small intestine, predominantly in the cytoplasm of enterocytes and goblet cells throughout the villi. XO protein expression is maximal in cells located midway through the length of the villi. In the lactating mammary gland there is high activity in acinar cells with some staining in non-lactating mammary gland (Linder *et al.* 1999). Immunohistochemical staining showed no activity in human heart, lung and brain although other workers have expressed XO mRNA in these tissues (Xu *et al.* 1994). Human AO mRNA is expressed in liver, lung, kidney, pancreas, prostate, testis and ovary (Wright *et al.* 1995). The AO gene is highly expressed in glial cells of the human spinal cord but, unlike mouse mRNA, not in neurones (Berger *et al.* 1995). This discrepancy could be due to a genuine species variation in brain localisation or differences in sample preparation/hybridisation techniques. In the same study, very little expression of XO mRNA was detected in the spinal cord (Berger *et al.* 1995). Interestingly XO activity in human brain is increased in tumour tissue (Kökoglu *et al.* 1990) and in rat olfactory cortex after systemic administration of the excitotoxin, kainic acid (Battelli *et al.* 1995).

Species variation in molybdenum hydroxylase activity

The molybdenum hydroxylases are found in most organisms throughout the animal kingdom. In 1974 Krenitsky *et al.*, on the basis of their functional and structural similarities, proposed that AO and XO evolved from a common primitive precursor whose gene(s) underwent duplication and subsequent divergent evolution. Recent studies by Terao *et al.* (1998) indicated a recent duplication event as intron/exon boundaries in human AO and XO were almost identical. Substrate specificity and kinetic properties of XO show less variation between species and tissues than AO (Beedham 1998). This is reflected in the conservation of XO gene sequences whereas there is less recognition between AO cDNAs from various animal species (Terao *et al.* 1998). Mammalian AO gene structures are similar to reptiles and birds whereas they diverge from that of insects and amphibians (Terao *et al.* 1998) and although plant and mammalian AOs are structurally related, there is little commonality between amino acid sequences of plants and mammalian AO (Kurosaki *et al.* 1999).

Despite the similarities between mammalian cDNAs and amino acid sequence (Kurosaki *et al.* 1999; Wright *et al.* 1999a) there is still a marked species variation in protein expression and catalytic activity of AO; this has been extensively reviewed by

Krenitsky *et al*. (1974), Moriwaki *et al*. (1998a, 1999) and Beedham (1985, 1987, 1998). Of particular interest in drug metabolism is the variation in enzyme activity in those species most widely used for pre-clinical trials, i.e. dog and rat.

AO ACTIVITY IN DOG AND RAT LIVER

AO activity, measured with a variety of substrates, is very low in dog liver (Rodrigues 1994; Krenitsky *et al*. 1974; Kitamura *et al*. 1999c). Minimal AO activity *in vitro* is mirrored by *in vivo* studies with carbazeran, a potent inotropic agent, in dog. Carbazeran undergoes rapid inactivation in baboon and humans catalysed by AO (Figure 5.4), but this route is absent in dogs (Kaye *et al*. 1984, 1985).

The existence of a marked variation/or lack of AO activity in Sprague–Dawley rats has been noted with many uncharged and charged heterocyclic substrates and aldehydes (Stanlovic and Chaykin 1971; Beedham *et al*. 1987a; Rashidi *et al.* 1997; Sugihara et al. 1995). Striking variations have also been noted in Wistar rats by Gluecksohn–Waelsch *et al*. (1967), who found appreciable AO activity towards N^1-methylnicotinamide in only 36 out of 76 animals. We have recently shown that functional enzyme was absent in 60% of Sprague–Dawley rats (Beedham 1998). Low and variable methotrexate-hydroxylating activity has led to conflicting reports relating to *in vivo* biotransformation in rats (Kitamura *et al*. 1999c).

Recent studies with Zaleplon, a sedative hypnotic, illustrate the marked species differences in AO activity. Zaleplon is metabolised by two competing pathways, 5-oxidation catalysed by AO and N-dealkylation mediated by cytochrome P450 isozymes (Figure 5.6). In mouse, rat and dog, where AO activity is low, the major metabolite is N-desethylzaleplon whereas in monkeys and humans, 5-oxozaleplon is formed (Chaudhary *et al.* 1994; Kawashima *et al*. 1999).

Superimposed on inter-animal variation there are also noticeable strain differences in both rats and mice. Sugihara *et al*. (1995) and Kitamura *et al*. (1999b) demonstrated 63- to 104-fold variation in AO-activity in 12 rat strains using the substrates, benzaldehyde and methotrexate respectively. Highest activity was obtained with SEA:SD rats and lowest with WKA/SEA rats. Expressed protein levels appear to correlate with catalytic activity and K_m values did not vary significantly among the different strains. In a separate study, Kunieda *et al.* (1999) distinguished 11 rat strains with high AO activity and 9 strains with no detectable activity. These authors mapped the AO locus in rats to chromosome 9, which is homologous to mouse chromosome 1 and human chromosome 2q. This is consistent with the loci for AO in the two latter species (Holmes 1979; Berger *et al*.1995). Hybrid crosses between 'high' and 'null' strains apparently showed intermediate activity indicating codominant alleles (Kunieda *et al.* 1999).

Factors affecting molybdenum hydroxylase activity *in vivo*

HORMONAL REGULATION OF AO ACTIVITY

It has long been recognised that hepatic AO activity is two- to four-fold higher in male than female mice and that AO levels may be controlled by androgenic and oestro-

genic hormones (Huff and Chaykin 1967, 1968; Gluecksohn-Waelsh *et al.* 1967). Furthermore, in mouse and rat, kinetically distinct AO forms are expressed in male and female liver (Yoshihara and Tatsumi 1997b; Wright *et al.* 1999a) and in both species significantly lower K_m values were obtained for substrate oxidation using the male enzyme compared to the female enzyme.

Treatment of female mice with testosterone proprionate increased hepatic AO activity which is associated with a decrease in the K_m value to the male type (Yoshihara and Tatsumi 1997a,b). In contrast, administration of oestradiol diproprionate significantly decreased enzyme levels in adult male mice (Ventura and Dachtler 1981; Yoshihara and Tatsumi 1997a). Oestradiol is a potent *in vitro* inhibitor of AO (Table 5.2) (Rajagopalan *et al.* 1962; Rashidi *et al.* 1995). Yoshihara and Tatsumi (1997a) have also shown that modulators of growth hormone, such as monosodium glutamate, also influence AO activity and they proposed that, in mice, pituitary growth hormone may be a major regulatory factor of gender differences in hepatic AO activity.

Kurosaki *et al.* (1999) compared AO mRNA, immunoreactive protein and catalytic activity in control male and female mice with those treated with testosterone proprionate. In concordance with previous reports (Yoshihara and Tatsumi 1997a,b), enzyme activity and protein levels were much higher in male than female liver but this was not correlated with gene expression as basal mRNA levels were similar in both sexes. Testosterone treatment increased the AO transcipt around three-fold with significant increases in protein levels/catalytic activity to male levels. Yoshihara and Tasumi (1997b) have suggested that different isozymes are present in male and female mouse liver. It should be noted that gender differences in AO activity appear to be restricted to liver as they are not observed in mouse lung, brain and spinal cord (Kurosaki *et al.* 1999). Furthermore, sex-specific differences may be restricted to mouse liver with little relevance to drug metabolism in humans.

Under saturating substrate conditions, we have found that AO activity does not vary significantly between males and females in guinea pig or rat liver (Beedham *et al.* 1987b; Rashidi 1996). On the other hand, Wright *et al.* (1999a) have shown that rat female hepatic AO exhibits kinetic characteristics distinct from male enzyme although results indicate that both forms are coded by one gene. They posit that male and female rat liver AO are interconverted by redox manipulation of the thiol:disulphide potential of a single protein. However, as only 40% of Sprague–Dawley rats show high AO activity (Rashidi 1996; Beedham 1998) the changes observed by Wright *et al.* (1999a) may reflect intra-strain variation rather that inter-sex differences.

Limited clinical data indicate that the pharmacokinetics of penciclovir, resulting from AO-catalysed oxidation, are similar in male and female volunteers (Pratt *et al.* 1994).

HORMONAL REGULATION OF XO ACTIVITY

Regulation of XO in mice appears to differ from that of AO. Yoshihara and Tatsumi (1997a) found, in the same animals that showed changes in AO activity, XO activity was constant and not affected by testosterone administration. Likewise, guinea pig liver XO did not vary significantly between males and females (Beedham *et al.* 1987b)

but results on rat liver XO are contradictory. Levinson (Levinson and Chalker 1980; Decker and Levinson 1982) reported androgen-dependent XO activity in rat liver and we have found that mean XO activity is higher in males (Rashidi 1996). However, in AO-active rats there was no difference in XO activity (see above).

There is also conflicting evidence about gender effects on human XO which may be due to different assay methods or other contributing factors. Guerciolini *et al.* (1991) used a direct, sensitive radiochemical assay to measure XO activity in liver samples from patients undergoing partial hepactectomy or open liver biopsy. XO activity in these samples was found to be significantly higher in male (1.43 $\pm$ 0.43 nmol uric acid formed/h per gm tissue) than female liver (1.05 $\pm$ 0.38 nmol uric acid formed/h per gm tissue). In contrast Relling *et al.* (1992), using caffeine metabolite ratios, reported significantly higher XO activity in female liver whereas other studies have found no significant gender differences (Kalow and Tang 1991; Vistisen *et al.* 1992; Chung *et al.* 2000). Nevertheless, boys are able to tolerate higher 6-mercaptopurine doses (Hale and Lilleyman 1991) but experience higher treatment failure rates than girls (Chessells *et al.* 1995). This would be consistent with higher oxidation rates to 6-thiouric acid by XO (Figure 5.8) which would switch the metabolic pathway away from activation to the inosine nucleotide.

The validity of using caffeine as an indicator of XO activity has been tested using allopurinol to inhibit caffeine metabolism and by comparing caffeine metabolite ratios with urinary concentration ratios of uric acid to xanthine and hypoxanthine (Grant *et al.* 1986). Caffeine is sequentially metabolised by CYP1A2, N-acetyltransferase and/or XO with a minor contribution from CYP2A6 (Figure 5.18) (Hamelin *et al.* 1994).

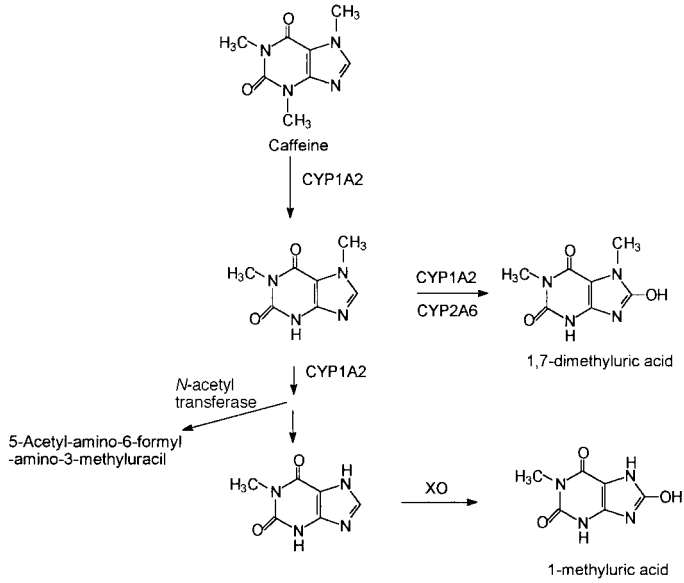


Figure 5.18 Major metabolic pathways of caffeine (Relling *et al.* 1992; Hamelin *et al.* 1994).

Measurement of metabolite ratios in urine arising from the intake of caffeine-containing beverages is a non-invasive method of phenotyping large populations. Nevertheless, results from caffeine studies are not always in agreement as metabolite ratios may be affected by caffeine intake and may not reflect changes in a single enzyme.

ETHNIC DIFFERENCES

Distribution of XO activity appears to be Gaussian (Grant *et al.* 1983; Guerciolini *et al.* 1991; Vistisen *et al.* 1992), although some studies indicate marked interindividual variation in some ethnic groups. Guerciolini *et al.* (1991) reported a 3.5- to 3.9-fold range in hepatic XO activity whereas Chung *et al.* (2000) have recently found up to nine-fold variation in caffeine metabolite ratios in Koreans. In the earlier study, probit analysis suggested that there may be a low activity subgroup in both males and females. In addition, ethnic differences in XO activity are indicated because XO indices were lower in black Americans than white caucasians, with 1% of population having very low XO activity all of whom were black American males (Relling *et al.* 1992). Information on the distribution of AO is not yet available.

EFFECT OF AGE OF MOLYBDENUM HYDROXYLASE ACTIVITY

There is no correlation between age and XO activity using either direct of indirect indicators of enzyme activity (Guerciolini *et al.*1991; Relling *et al.*, 1992; Chung *et al.* 2000). Total clearance of allopurinol does not differ in elderly subjects compared to young controls although clearance of its active metabolite, oxipurinol is significantly reduced (Turnheim *et al.* 1999). Elimination of allopurinol is predominantly via metabolism whereas oxipurinol is cleared by renal excretion. Although Turnheim *et al.* (1999) have suggested that allopurinol metabolism is a function of XO activity, AO is thought to be primarily responsible for the oxidation of allopurinol to oxipurinol (Reiter *et al.* 1990; Moriwaki *et al.* 1993a) and thus it would appear that neither molybdenum hydroxylase varies in elderly volunteers. Similarly, studies with famciclovir in elderly volunteers and patients have shown that similar amounts of penciclovir, the AO-generated active metabolite (Figure 5.7) are excreted in the urine although renal clearance of penciclovir was slightly lower in elderly subjects (Fowles *et al.* 1992; Perry and Wagstaff 1995). As creatinine clearance was also decreased in elderly patients, the changes in penciclovir pharmacokinetics were thought to be due to age-dependent decreases in renal function, which is also observed with oxipurinol excretion.

Hamelin *et al.* (1994), using caffeine as a probe substrate, found that XO activity was higher in children with cystic fibrosis than age-matched volunteers. This may have implication in drug disposition in this population but may be more significant in the pathogenesis of cystic fibrosis due to an increase in the production of ROS.

Genetic factors and molecular biology

AO and XO are located on the same chromosome in humans at a short distance from each other (Terao *et al.* 1998). The candidate gene for AO maps on chromo-

some 2q33-q35 (Wright *et al*. 1997; Terao *et al*. 1998) whereas the locus for XO is 2p22 or 2p23 (Ichida *et al*. 1993; Xu *et al*. 1994; Berger *et al*. 1995). Lack of XO is a rare autosomal recessive disorder known as hereditary xanthinuria (Shibutani *et al*. 1999). It is characterised by hypouricaemia, hypouricosuria and xanthinuria and has an incidence of about 1 in 70 000 (Harkness *et al*. 1986). Classical xanthinuria was first reported in 1954 by Dent and Philport (1954) but, on the basis of results obtained from xanthinuric patients with allopurinol, has been more recently classified into two or three subtypes. Classical type 1 xanthinuria is characterised by XO deficiency whereas in classical type 2 xanthinuria both XO and AO are lacking (Reiter *et al*. 1990). Consequently, allopurinol is converted to oxipurinol via AO in type 1 patients whereas type 2 patients are unable to form oxipurinol (Reiter *et al.* 1990 and references therein). Ishida and co-workers have identified a nonsense mutation and deletion of the XO gene in patients with classical type 1 xanthinuria confirming that the primary genetic defect in this subgroup is the XO gene (Ichida *et al*. 1997; Levartovsky *et al*. 2000). In contrast, an abnormality in the molybdenum cofactor that is required for both enzymes is thought to be the cause of classical type 2 xanthinuria (Ichida *et al*. 1998). To date, there are no reports of a single deficiency in aldehyde oxidase.

AO and XO activities are also lacking in patients manifesting Molybdenum Cofactor Deficiency (Reiss 2000). This is also a rare autosomal recessive disorder that, unlike xanthinuria which is relatively benign, leads to severe neurological symptoms and an early childhood death. It is caused by defects in the biosynthesis of the molybdopterin cofactor which is required for AO, XO and sulphite oxidase. Although hypouricaemia is observed in Molybdenum Cofactor Deficiency, the pathogenesis of the clinical features resemble those seen in isolated sulphite oxidase deficiency and thus may not be related to AO or XO (Shalata *et al*. 1998, 2000).

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