Kinetics and specificity of guinea pig liver aldehyde oxidase and bovine milk xanthine oxidase towards substituted benzaldehydes

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Molybdenum-containing enzymes, aldehyde oxidase and xanthine oxidase, are important in the oxidation of N-heterocyclic xenobiotics. However, the role of these enzymes in the oxidation of drug-derived aldehydes has not been established.

The present investigation describes the interaction of eleven structurally related benzaldehydes with guinea pig liver aldehyde oxidase and bovine milk xanthine oxidase, since they have similar substrate specificity to human molybdenum hydroxylases. The compounds under test included mono-hydroxy and mono-methoxy benzaldehydes as well as 3,4-dihydroxy-, 3-hydroxy-4-methoxy-, 4-hydroxy-3-methoxy-, and 3,4-dimethoxy-benzaldehydes. In addition, various amines and catechols were tested with the molybdenum hydroxylases as inhibitors of benzaldehyde oxidation.

The kinetic constants have shown that hydroxy-, and methoxy-benzaldehydes are excellent substrates for aldehyde oxidase (K_m values $5{\times}10^{-6}$ M to $1{\times}10^{-5}$ M) with lower affinities for xanthine oxidase (K_m values around 10^{-4} M). Therefore, aldehyde oxidase activity may be a significant factor in the oxidation of the aromatic aldehydes generated from amines and alkyl benzenes during drug metabolism. Compounds with a 3-methoxy group showed relatively high V_{max} values with aldehyde oxidase, whereas the presence of a 3-hydroxy group resulted in minimal V_{max} values or no reaction. In addition, amines acted as weak inhibitors, whereas catechols had a more pronounced inhibitory effect on the aldehyde oxidase activity. It is therefore possible that aldehyde oxidase may be critical in the oxidation of the analogous phenylacetaldehydes derived from dopamine and noradrenaline.

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Aldehydes are widely distributed in nature. They constitute a group of relatively reactive organic compounds which occur as natural constituents in a wide variety of foods. More than 300 aldehydes have been identified in foods and food components and are widely used as flavouring agents (Feron *et al.*, 1991; Lindahl, 1992).

Most aldehydes are irritants producing tissue damage at the site of application, and many possess genotoxic activity (Dellarco, 1988; Ma & Harris, 1988). Some aldehydes are potentially carcinogenic in several animal species, whereas others have limited or no carcinogenicity (Feron *et al.*, 1991).

Although aldehydes are not typically found in any measurable concentration in cells, many compounds (these include alcohols, amines and hydrocarbons) are ultimately converted, *via* an aldehyde intermediate, to the corresponding acid.

Even though only a few drugs contain aldehyde groups in their structures, there are many drugs with an amino group attached to an unsubstituted methylene carbon which can be transformed into aldehydes *via* the action of monoamine oxidase. Exposure to exogenous aldehydes can also occur through the ingestion of metabolic precursors such as primary alcohols. Another common source of an aldehydic group is the hydroxylation of an aromatic methyl group to an alcohol, which subsequently undergoes oxidation, such as the oral hypoglycaemic agent tolbutamide, which is used for the treatment of diabetes mellitus (Washio *et al.*, 1993).

Aldehydes are also produced in the body *via* oxidative deamination of compounds such as biogenic amines, catalyzed by the action of monoamine oxidase. For example, homovanillamine is deaminated to homovanillyl aldehyde (Beedham *et al.*, 1995b). In addition, nearly all of the common α-amino acids are converted to reactive aldehydes by the enzyme myeloperoxidase (Hazen *et al.*, 1998).

Biogenic and xenobiotic aldehydes can either be oxidized to the corresponding acids, which may be catalysed by aldehyde dehydrogenase (EC 1.2.1.3, aldehyde:NAD$^+$ oxidoreductase), aldehyde oxidase (EC 1.2.3.1, aldehyde:oxygen oxidoreductase) and/or xanthine oxidase (EC 1.2.3.2, xanthine:oxygen oxidoreductase), or reduced by aldehyde reductase and/or alcohol dehydrogenase to the corresponding alcohols (Fig. 1). Although some aldehydes may be reduced to alcohols *in vivo*, the main metabolic routes are conversion to acids.
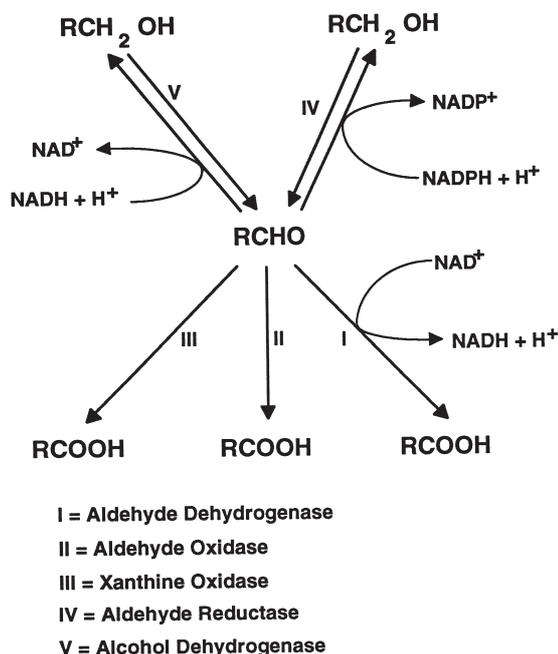

I = Aldehyde Dehydrogenase
II = Aldehyde Oxidase
III = Xanthine Oxidase
IV = Aldehyde Reductase
V = Alcohol Dehydrogenase

Figure 1. The pathways of aldehyde metabolism.

The molybdenum hydroxylases aldehyde oxidase and xanthine oxidase catalyse the oxidation of a wide range of N-heterocycles and aldehydes (Krenitsky *et al.*, 1972; Beedham, 1985; Beedham *et al.*, 1995a). Most of the reports in the literature have utilized heterocycles as substrates rather than aldehydes and many studies have been carried out with rabbit liver aldehyde oxidase, which has different properties than human liver aldehyde oxidase (Johns, 1967; Beedham, 1987). Aldehyde oxidase activity is predominant in the liver (Sasaki *et al.*, 1983; Beedham *et al.*, 1987), whereas xanthine oxidase activity is present at high levels in the lactating

mammary gland (Bruder *et al.*, 1982) and primarily in cow's milk but not in human milk (Modi *et al.*, 1959). Although these enzymes are similar in structure and molecular properties, they differ in substrate specificity (Krenitsky *et al.*, 1972). While the substrate specificity towards aromatic heterocycles has been extensively investigated, very few systematic studies on the enzymatic oxidation of aldehydes are found in the literature (Booth, 1938; Gordon *et al.*, 1940; Pelsy & Klibanov, 1983).

The present study examines the interaction of eleven structurally related benzaldehydes with guinea pig liver aldehyde oxidase and bovine milk xanthine oxidase, as these enzymes have similar substrate specificity to human molybdenum hydroxylases (Beedham *et al.*, 1987a; Critchley, 1989). The compounds under test included mono-hydroxy-, and mono-methoxy-benzaldehydes, as well as 3,4-dihydroxy-, 3-hydroxy-4-methoxy-, 4-hydroxy-3-methoxy-, and 3,4-dimethoxy-benzaldehydes. In addition, a number of amines and catechols were tested with guinea pig liver aldehyde oxidase as inhibitors of benzaldehyde oxidation.

MATERIALS AND METHODS

Animals. The animals used in this study were Dunkin-Hartley guinea pigs (450–950 g). They were fed with FD1 pellets supplemented with ascorbic acid and received hay three times weekly. All animals were allowed food and water *ad libitum* and maintained in a strictly controlled temperature; $18 \pm 1°C$. Humidity was kept at 50% and the lighting cycle was 07.00–19.00 h light and 19.00–07.00 h dark.

Chemicals. 4-Hydroxy-3-methoxybenzaldehyde (vanillin), 3,4-dihydroxybenzaldehyde (protocatechuic aldehyde), 3-hydroxy-4-methoxybenzaldehyde (isovanillin), 3-methoxybenzaldehyde (3-anisaldehyde), 2-phenylethylamine hydrochloride, 3,4-dimethoxy-2-phenyl-

ethylamine (homoveratrylamine), 3,4-dihydroxyphenylethylamine (dopamine), 3,4-dihydroxyphenylethanolamine (noradrenaline), L-3,4-dihydroxyphenylalanine (L-DOPA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were supplied from Sigma Chemical Company Ltd. Benzaldehyde, 2-hydroxybenzaldehyde (salicylaldehyde), 3,4-dimethoxybenzaldehyde, 4-hydroxybenzaldehyde, 2-methoxybenzaldehyde (2-anisaldehyde), 4-methoxybenzaldehyde (4-anisaldehyde), phthalazine, 4-hydroxy-3-methoxy-2-phenylethylamine (homovanillamine), and phenanthridine were purchased from Aldrich Chemical Co. Ltd. 3-Hydroxybenzaldehyde was from Ralph Emanuel, 1,2-dihydroxybenzene (catechol) from BDH Chemicals Ltd, xanthine (pure) from Koch-Light Laboratories Ltd, Coomassie Blue G-250 from Pierce, and 2-methylphthalaziniun iodide was synthesized according to procedures of Smith & Otremba (1962).

Enzymes. Xanthine oxidase (grade I) was obtained from Sigma Chemical Company Ltd: from bovine buttermilk (EC 1.2.3.22), 0.47 units/g protein, 38 mg protein/ml (biuret).

Preparation of partially purified aldehyde oxidase from guinea pig liver. Guinea pigs were killed by cervical dislocation and their livers were placed in 3–4 volumes of ice-cold isotonic potassium chloride solution (1.15% KCl, w/v). They were then homogenized on ice in a homogenizer fitted with a Teflon pestle.

The homogenate was heated at 55–57°C for 11 min and then centrifuged at $15\,000 \times \boldsymbol{g}$ for 45 min at 4°C. The resulting clear red supernatant was filtered through glass wool and then solid ammonium sulphate was added to give 50% saturated solution (35.3 g/100 ml at 4°C).

The resulting suspension was centrifuged at $6000 \times \boldsymbol{g}$ for 20 min at 4°C and the precipitate re-dissolved in 5 ml of 1 mM EDTA per animal liver. The enzyme was stored in liquid nitrogen in the form of small beads until needed.

Spectrophotometric determination of enzyme activity. All kinetic measurements and UV spectra were determined using a Pye-Unicam SP8-250 UV/Visible Spectrophotometer fitted with a Pye-Unicam cell temperature controller at 37°C.

Aldehyde oxidase activity was routinely estimated using potassiun ferricyanide (1 mM) as the electron acceptor, at 420 nm, using a heterocyclic substrate such as phthalazine. Xanthine oxidase activity was checked with xanthine, at 295 nm, and molecular oxygen as the electron acceptor.

Protein content estimation in aldehyde oxidase. The protein content in aldehyde oxidase was determined using Coomassie Blue dye reagent and bovine serum albumin as the standard, measured at 595 nm, according to the method of Bradford (1976).

Determination of kinetic constants for aldehyde oxidase. Kinetic constants were determined using aldehyde oxidase which was prepared from three animal livers. All assays were carried out in 67 mM Sörensen's phosphate buffer, pH 7.0, containing 0.1 mM EDTA, varying concentrations of substrate, potassium ferricyanide (1 mM), in a final volume of 3 ml and sufficient enzyme to produce measurable rates over the range of substrate concentrations under study.

Michaelis–Menten constants were determined by measuring the initial oxidation rates from a minimum ten different substrate concentrations over the first 2–5 min, following spectrophotometrically the decrease in absorbance at 420 nm. All aldehydes were initially tested for non-enzymatic oxidation and/or interaction with potassium ferricyanide.

Since many of the benzaldehydes interacted with potassium ferricyanide, the oxidation rates of these substrates were monitored directly. This was achieved by determining the UV spectra of aldehydes and their acid products, which were scanned individually in the range between 200–400 nm. Absorbance maxima were chosen in the UV spectrum of

the aldehydes, at which wavelength the corresponding acid had minimal absorbance at a similar concentration. The molar absorption of each aldehyde was calculated at the specified wavelength.

The wavelengths chosen for measurement of the initial oxidation rates for each aldehyde, using molecular oxygen as the electron acceptor, and the corresponding molar absorption are presented in Table 1.

Therefore, the oxidation rates of benzaldehydes were measured at the chosen wavelength under similar conditions as with potassium ferricyanide assay. All initial rates measured were found to be linear over the first 2–3 min of reaction.

Assays were validated by incubating each aldehyde with aldehyde oxidase and scanning the resultant UV spectrum from 200–400 nm at various time intervals until acid formation was maximal. In each case the final UV spectra corresponded to that of the acid. This is illustrated in Fig. 2 for 3-methoxybenzaldehyde.

The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined from the 'Lineweaver-Burk' plot of $1/V$ *versus* $1/[S]$. The line of the best fit through the points was calculated using simple linear regression by the least squares method. The 'coefficient of determination' was used to express the goodness of fit of the line. The substrate efficiency (K_s), was calculated from the formula: $K_s = V_{max}/K_m$.

Determination of kinetic constants for xanthine oxidase. The rates of aldehyde oxidation by xanthine oxidase were measured with molecular oxygen as electron acceptor and at the same wavelengths as for aldehyde oxidase.

Determination of the mode of inhibition and inhibitor constants for molybdenum hydroxylase inhibitors. The effect of inhibitors upon the oxidation rate of a substrate was also investigated spectrophotometrically. Compounds which showed no reaction as substrates by aldehyde oxidase were tested as in-

Table 1. Wavelengths and molar absorption used for the measurement of the enzymatic oxidation of substituted benzaldehydes.

Substrate	Wavelength (nm)	Molar absorption, ε (M^{-1} cm^{-1})
Benzaldehyde	249	10083
2-Hydroxybenzaldehyde	255	9728
3-Hydroxybenzaldehyde	255	9813
4-Hydroxybenzaldehyde	290	12917
2-Methoxybenzaldehyde	256	11042
3-Methoxybenzaldehyde	256	10313
4-Methoxybenzaldehyde	284	17130
3,4-Dihydroxybenzaldehyde	330	8499
3-Hydroxy-4-methoxybenzaldehyde	230	6500
4-Hydroxy-3-methoxybenzaldehyde	310	8854
3,4-Dimethoxybenzaldehyde	310	9333

UV spectra of aldehydes and their acid products were scanned individually in the range of 200–400 nm. The absorbance maxima were chosen in the UV spectrum of the aldehydes, at which wavelength the corresponding acid had minimal absorbance at a similar concentration.

hibitors of benzaldehyde or 2-hydroxybenzaldehyde oxidation. The rate of oxidation of at least ten varying concentrations of 2-hydroxybenzaldehyde was monitored in the
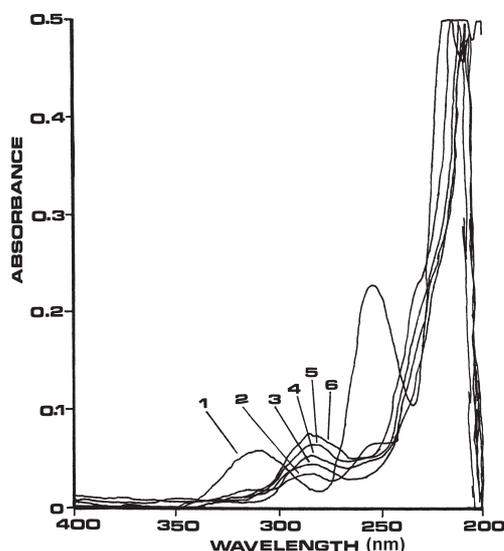

Figure 2. UV spectra of oxidation of 3-methoxybenzaldehyde by aldehyde oxidase at consecutive time intervals.

3-Methoxybenzaldehyde (0.024 mM) was incubated with 0.015 ml aldehyde oxidase (18.6 mg/ml) in 3 ml 67 mM Sörensen's phosphate buffer, pH 7.0, containing 0.1 mM EDTA, at consecutive time intervals (1, absence of enzyme; 2, 0 min; 3, 5 min; 4, 10 min; 5, 15 min; 6, 20 min).

presence or absence of two concentrations of an inhibitor, [I].

The type of inhibition was determined by examination of double reciprocal plots of both non-inhibited and inhibited reactions and the inhibitor constant (K_i) was calculated by the use of information derived from these plots.

RESULTS AND DISCUSSION

Kinetic constants for the oxidation of substituted benzaldehydes by guinea pig liver aldehyde oxidase

The K_m, V_{max} and K_s values calculated for aldehyde oxidase with the heterocyclic substrate phthalazine, using ferricyanide as an electron acceptor, were found to be 0.089 mM, 0.44 μmol/min per mg protein and 5.0 ml/min per mg protein, respectively. These kinetic constants are similar to those reported previously for phthalazine (Beedham et al., 1990; Critchley et al., 1992).

All kinetic plots showed substrate inhibition at concentrations of around $10 \times K_m$ value. Substrate inhibition was also noticed in the oxidation of 2-hydroxybenzaldehyde (50 $\times$
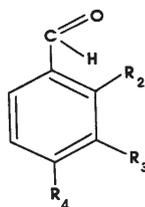
K_m value) with rabbit liver aldehyde oxidase (Rajagopalan & Handler, 1964) and 4-hydroxy-3-methoxybenzaldehyde at concentrations greater than 5 mM with aldehyde oxidase from *Streptomyces viridosporus* T7A (Wilkof *et al.*, 1984). However, this phenomenon is not only restricted to aldehydes, but also to uncharged substrates such as phthalazine (Coughlan, 1980) and quinoline which exhibit marked substrate inhibition (Rajagopalan & Handler, 1964). Excess substrate may cause an over reduction of Mo(III) which results in a slower reoxidation to Mo(VI) (Coughlan, 1980). In contrast, quaternary substrates such as *N*-methylquinolinium and *N*-ethylquinolinium do not show substrate inhibition with aldehyde oxidase (Taylor *et al.*, 1984).

Most of the substituted benzaldehydes were found to be excellent substrates of hepatic guinea pig aldehyde oxidase (Table 2). The lowest K_m values were obtained with 2-methoxy-, 3-hydroxy-, and 4-hydroxy-benzaldehydes and the best substrate for guinea pig aldehyde oxidase, in terms of substrate efficiency (K_s), is 2-methoxybenzaldehyde. Relatively low V_{max} values were obtained with 3-hydroxy-, and 4-hydroxy-benzaldehydes.

Benzaldehyde oxidation has also been studied with human liver aldehyde oxidase (Johns, 1967) under similar conditions to those employed in the present study. Johns found the K_m for the human liver enzyme to be of the same order as the K_m value reported here for guinea pig liver aldehyde oxidase. This is further evidence of the similarity in substrate specificity between guinea pig and human liver aldehyde oxidase (Krenitsky *et al.*, 1986; Beedham *et al.*, 1987a). Although absolute oxidation rates were not reported by Johns (1967), benzaldehyde had higher reaction velocities than the aliphatic aldehydes tested as substrates for the human liver aldehyde oxidase.

4-Hydroxy-3-methoxybenzaldehyde was found to be an excellent substrate for guinea pig liver aldehyde oxidase with K_m value (Table 2) similar to that reported by Wilkof *et al.*

Table 2. Kinetic constants for the oxidation of substituted benzaldehydes by guinea pig liver aldehyde oxidase.

Substituent			K_m*	V_{max}	$K_s=(V_{max}/K_m)$
R_2	R_3	R_4	(mM)	(μmol/min per mg)	(ml/min per mg)
H	H	H	0.019	0.34	18
OH	H	H	0.02	0.30	15
H	OH	H	0.005	0.05	10
H	H	OH	0.008	0.12	15
OCH$_3$	H	H	0.004	0.33	73
H	OCH$_3$	H	0.02	0.38	19
H	H	OCH$_3$	0.013	0.28	22
H	OH	OH	I**		
H	OH	OCH$_3$	I**		
H	OCH$_3$	OH	0.029	0.45	17
H	OCH$_3$	OCH$_3$	0.019	0.38	21

The oxidation rates of the substituted benzaldehydes by aldehyde oxidase were measured spectrophotometrically at 37°C with molecular oxygen as an acceptor. For details see Materials and Methods. All correlation coefficients (r) > 0.998; *n = 2; **I = inhibitor.

(1984) for both soluble and immobilised aldehyde oxidase prepared from *S. viridosporus* T7A. 4-Hydroxy-3-methoxybenzaldehyde has also been used to study species variation of aldehyde oxidase occurrence in 79 species. Wurzinger & Hartestein (1974) found 4-hydroxy-3-methoxybenzaldehyde to be a substrate of aldehyde oxidase from mollusks, crustaceans and insects. Aldehyde oxidase activity was not found in birds, but it was observed in four other classes of vertebrates: osteichthyes, amphibia, reptilia and mammalia (Sprague-Dawley rat liver and DBA mouse liver).

The K_m value obtained for 2-hydroxybenzaldehyde with guinea pig hepatic aldehyde oxidase is markedly lower than the K_m value of 0.15 mM calculated by Rajagopalan & Handler (1964) for this compound using rabbit liver aldehyde oxidase. However, it has been noted previously that the substrate specificity of the lapine liver enzyme is quite different to that of guinea pig and human aldehyde oxidase (Johns, 1967; Beedham *et al.*, 1987a; Beedham *et al.*, 1990).

In general, hydroxy-, and methoxy-benzaldehydes are more efficient substrates for guinea pig liver aldehyde oxidase (Table 2) than most heterocyclic substrates such as phthalazine (K_m= 0.052 mM, V_{max}= 0.542 μmol/min per mg protein) (Beedham *et al.*, 1990), quinazoline (K_m= 0.031 mM, V_{max}= 0.639 μmol/min per mg protein) (Critchley, 1989) and *N*-methylquinolinium salts (K_m= 0.05 mM) (Taylor *et al.*, 1984). This is due to higher affinities of the enzyme rather than higher V_{max} values. K_m values for heterocyclic substrates tend to range from 0.02 mM to 0.01 mM, whereas those for these aromatic aldehydes are between 0.004 mM to 0.03 mM. This may be due to methodological differences, for example the use of different electron acceptors. The oxidation rates of the aldehydes in this study were measured by using molecular oxygen as the electron acceptor whereas those of heterocyclic substrates with an artificial electron acceptor, ferricyanide.

Reoxidation of aldehyde oxidase occurs at different redox centers depending on the electron acceptor used. Oxygen reacts at the flavin center (FAD) of aldehyde oxidase, whereas ferricyanide acts as the oxidizing substrate at an iron/sulphur center of the enzyme (Coughlan & Ni Fhaolain, 1979). However, it was possible to determine the kinetic constants for one of the aldehydes, 4-hydroxy-3-methoxybenzaldehyde, using the same enzyme preparation with either molecular oxygen or ferricyanide as electron acceptors. The K_m and V_{max} values calculated for this compound were 0.029 mM and 0.45 μmol/min per mg protein, respectively, when molecular oxygen was used as the electron acceptor, whereas in the presence of ferricyanide the values were 0.035 mM and 0.41 μmol/min per mg protein, respectively. This indicates that, for this group of compounds, the choice of electron acceptor does not have a significant effect on the kinetic constants. Similarly, uncharged substrates such as phthalazine and quinoline show only small variations in K_m values with different electron acceptors (Taylor *et al.*, 1984).

In contrast to most of the other aldehydes, 3-hydroxy-4-methoxy-, and 3,4-dihydroxybenzaldehydes did not react with guinea pig liver aldehyde oxidase, although the other two di-substituted benzaldehydes, 4-hydroxy-3-methoxy-, and 3,4-dimethoxy-benzaldehydes, were found to be reasonable substrates (Table 2). These compounds were found to be inhibitors of aldehyde oxidase from guinea pig liver (Panoutsopoulos, 1994; Beedham *et al.*, 1995b).

3-Hydroxy-4-methoxy-, and 3,4-dihydroxybenzaldehydes as inhibitors of aldehyde oxidation by guinea pig liver aldehyde oxidase

3-Hydroxy-4-methoxy-, and 3,4-dihydroxybenzaldehydes were tested as inhibitors of 2-hydroxybenzaldehyde oxidation (Table 3).

Table 3. The inhibitory effect of 3,4-dihydroxy-, and 3-hydroxy-4-methoxy-benzaldehydes on guinea pig liver aldehyde oxidase with 2-hydroxybenzaldehyde as substrate.

Inhibitors	Inhibitor concentration (mM)	Inhibition of 2-hydroxy-benzaldehyde oxidation* (%)
3-Hydroxy-4-methoxybenzaldehyde	0.1	87
3,4-Dihydroxybenzaldehyde	0.1	87

*Concentration of 2-hydroxybenzaldehyde = 0.1 mM

3-Hydroxy-4-methoxybenzaldehyde (Fig. 3) and 3,4-dihydroxy-benzaldehyde (Fig. 4) were both potent competitive inhibitors of 2-hydroxybenzaldehyde oxidation by aldehyde oxidase with K_i values of 6.64×10^{-4} mM (n = 2) and 1.57×10^{-4} mM (n = 2), respectively.

Johnson *et al.* (1985) reported an inhibitory effect of hydralazine on the oxidation of purine by rabbit, guinea pig and baboon liver aldehyde oxidase. Critchley (1989) tested the ef-

fect of hydralazine administration on guinea pig liver aldehyde oxidase. He found that the oxidation rate of phthalazine, phenanthridine and carbazeran was reduced to less than half that of control animals when the enzyme was prepared from hydralazine (10 mg/kg per day) treated guinea pigs. However, when xanthine was used as substrate there was no reduction in the oxidation rate. These results suggest that hydralazine acts as a potent inhibitor of aldehyde oxidase *in vivo* and *in vitro*, but does not have any inhibitory effect on xanthine oxidase.

Inhibition of aldehyde oxidase by amines and catechols

As inhibition of aldehyde oxidase appeared to be related to the presence of 3-hydroxy-, or 3,4-dihydroxy-substituents in the benzaldehydes, a number of amines and catechols were tested with guinea pig liver aldehyde oxidase (Table 4). However, the same benzaldehyde concentration could not always be employed in each case because (a) difficulties were encountered with measuring out small amounts of liquid and (b) the cumulative absorbance of both benzaldehyde and some of the inhibitors made spectrophotometric readings not possible.

Of all the compounds tested, 2-phenylethylamine, at 1 mM, was the weakest inhibitor showing only a marginal effect on benzaldehyde oxidation. 4-Hydroxy-3-methoxy-2-phenylethylamine was also found to be relatively ineffective as an inhibitor but
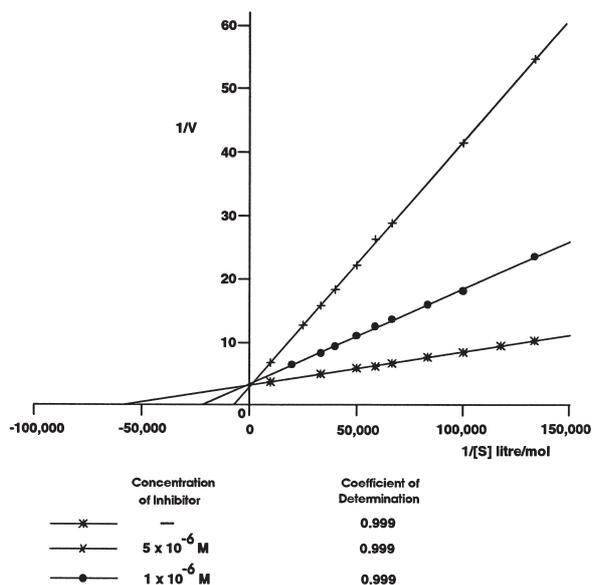

Figure 3. Lineweaver-Burk plots for determination of the inhibitor constant for 3-hydroxy-4-methoxybenzaldehyde (isovanillin) with guinea pig liver aldehyde oxidase.

The rate of oxidation of at least ten concentrations of 2-hydroxybenzaldehyde was monitored in the presence or absence of two concentrations of the inhibitor, [I], and measured at 37°C with molecular oxygen as electron acceptor at 255 nm.

3,4-dimethoxy-2-phenylethylamine inhibited benzaldehyde oxidation to a great extent.

The catecholamines, 3,4-dihydroxy-2-phenylethylamine (dopamine), 3,4-dihydroxy-phenylethanolamine (noradrenaline) and catechol (1,2-dihydroxybenzene), had the greatest effect on benzaldehyde oxidation. Thus at equimolar concentrations, noradrenaline (0.1 mM) inhibited the oxidation by 90%. As unsubstituted catechol inhibited

Alton, 1968), was only a weak inhibitor of aldehyde oxidase.

Inhibition studies of 2-hydroxybenzaldehyde oxidation showed dopamine to be a potent uncompetitive inhibitor of aldehyde oxidase with a K_i value of 0.0071 mM (Fig. 5).

Uncompetitive inhibition, as exhibited towards aldehyde oxidase, normally implies reaction of the inhibitor with the enzyme–substrate complex [ES] to produce a dead end

Table 4. The inhibitory effect of catechols and amines on guinea pig liver aldehyde oxidase with benzaldehyde as substrate.

Inhibitors	Inhibitor concentration (mM)	Inhibition of benzaldehyde oxidation (%)
2-Phenylethylamine*	1	10
	0.1	10
4-Hydroxy-3-methoxy-2-phenylethylamine*	1	55
	0.1	21
3,4-Dimethoxy-2-phenylethylamine*	1	100
	0.1	48
Dopamine**	1	100
	0.1	59
Noradrenaline**	1	100
	0.1	90
Catechol**	1	98
	0.1	64
L-Dopa**	1	94
	0.1	39
DOPAC***	1	27
	0.05	16

*Benzaldehyde concentration = 0.04 mM; **Benzaldehyde concentration = 0.1 mM; ***Benzaldehyde concentration = 0.8 mM.

benzaldehyde oxidation to a similar extent as that of dopamine, it would appear that the inhibition may be caused by the presence of a catechol group, rather than of the ethylamine side chain. This is supported by the results obtained with 2-phenylethylamine. L-DOPA appeared to be a less effective inhibitor of aldehyde oxidase than dopamine, catechol and noradrenaline. 3,4-Dihydroxyphenylacetic acid (DOPAC), one of the major metabolites of dopamine (Williams *et al.*, 1960; Goodall &

product [ESI], the inhibitor preventing the regeneration of the enzyme in a form capable of reacting directly with the substrate.

Inhibition of aldehyde oxidase by substituted catechols

In order to determine whether the inhibition observed with 3-hydroxy-4-methoxybenzaldehyde and 3,4-dihydroxybenzaldehyde, noradrenaline and dopamine was a general
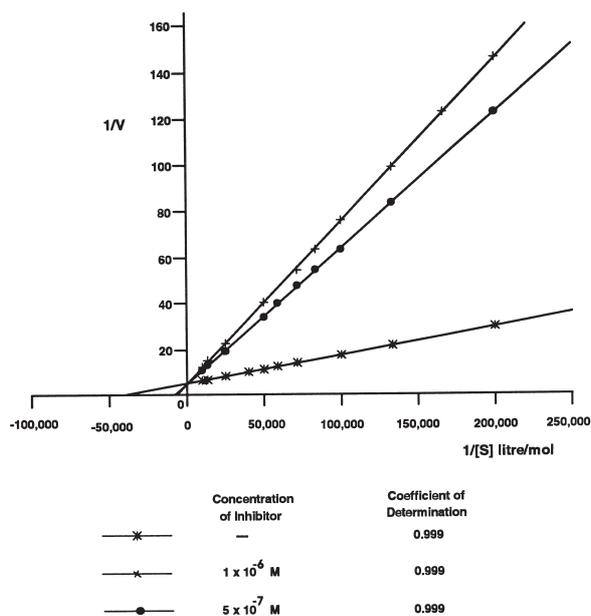
Figure 4. Lineweaver-Burk plots for determination of the inhibitor constant for 3,4-dihydroxy-benzaldehyde (protocatechuic aldehyde) with guinea pig liver aldehyde oxidase.

The rate of oxidation of at least ten concentrations of 2-hydroxy-benzaldehyde was monitored in the presence or absence of two concentrations of the inhibitor, [I], and measured at 37°C with O_2 as electron acceptor at 255 nm.
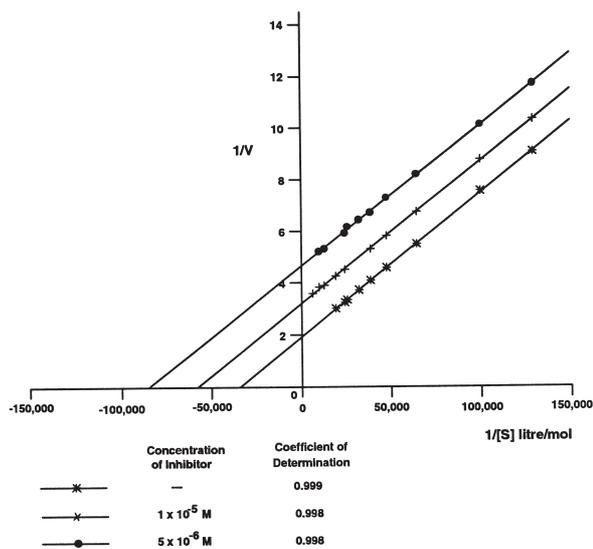
Figure 5. Lineweaver-Burk plots for determination of the inhibitor constant for dopamine with guinea pig liver aldehyde oxidase.

The rate of oxidation of at least ten concentrations of 2-hydroxybenzaldehyde was monitored in the presence or absence of two concentrations of the inhibitor, [I], and measured at 37°C with molecular oxygen as electron acceptor at 255 nm.

Table 5. The inhibitory effect of substituted catechols on guinea pig liver aldehyde oxidase with phenanthridine and *N*-methylphthalazinium chloride as substrates.

Inhibitors	Inhibitor concentration (mM)	Inhibition of substrate oxidation (%)	
		Phenanthridine*	*N*-methylphthalazinium**
3-Hydroxy-4-methoxybenzaldehyde	0.1	87	85
	0.01	17	34
	0.001	0	20
3,4-Dihydroxybenzaldehyde	0.1	96	§
	0.01	50	76
	0.001	3	64
Noradrenaline	1	91	§
	0.1	51	95
	0.01	17	32
	0.001	10	6
Dopamine	0.1	80	93
	0.01	19	32
	0.001	0	6

*Phenanthridine concentration = 0.05 mM (λ_{max} = 322 nm); **N*-Methylphthalazinium concentration = 0.05 mM (λ_{max} = 320 nm); molecular oxygen was used as an acceptor. §, Combined absorbance too high.

property of these compounds, or peculiar to the oxidation of aldehydes, these compounds were also tested against two N-heterocyclic substrates, namely phenanthridine and *N*-methylphthalazinium chloride.
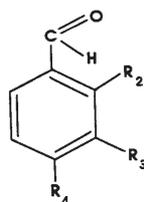
These two heterocyclic substrates were chosen for three reasons. Firstly, both phenanthridine (Stubley & Stell, 1980; Beedham, 1987) and *N*-methylphthalazinium (Critchley, 1989; Beedham *et al*., 1990) are relatively specific substrates for aldehyde oxidase. Secondly, phenanthridine is a typical uncharged heterocycle, whereas *N*-methylphthalazinium is a charged quaternary compound and these may bind at different sites. Thirdly, their use

(Table 3). 3,4-Dihydroxybenzaldehyde inhibited aldehyde oxidase with phenanthridine as substrate and, particularly, *N*-methylphthalazinium (Table 5) to a greater extent than that of 2-hydroxybenzaldehyde (Table 3).

The percentage inhibition of *N*-methylphthalazinium and benzaldehyde oxidations by noradrenaline were of similar magnitude, whereas the effect upon phenanthridine oxidation was less marked. Dopamine appeared to be an even more effective inhibitor of phenanthridine and *N*-methylphthalazinium oxidation than of benzaldehyde.

These results showed that 3-hydroxy-4-methoxybenzaldehyde, 3,4-dihydroxybenzalde-

Table 6. Kinetic constants for the oxidation of substituted benzaldehydes by bovine milk xanthine oxidase.

Substituent			K_m*	V_{max}	$K_s = (V_{max}/K_m)$
R_2	R_3	R_4	(mM)	(μmol/min per mg)	(ml/min per mg)
H	H	H	0.3	0.17	0.5
OH	H	H	0.19	0.67	3.5
H	OH	H	0.18	0.95	5.4
H	H	OH	0.17	3.29	19.8
OCH_3	H	H	0.19	0.10	0.5
H	OCH_3	H	0.17	0.59	3.4
H	H	OCH_3	0.17	0.17	1.0
H	OH	OH	0.039	0.40	10.0
H	OH	OCH_3	0.18	0.95	5.3
H	OCH_3	OH	0.12	0.27	1.3
H	OCH_3	OCH_3	0.68	0.22	0.3

The oxidation rates of substituted benzaldehydes by xanthine oxidase were measured spectrophotometrically at 37°C with molecular oxygen as an acceptor. For details see Materials and Methods. All correlation coefficients (r) > 0.998; *n = 2.

facilitates the experimental conditions, since substrate and product have different UV spectra making spectrophotometric measurements under oxygen possible.

3-Hydroxy-4-methoxybenzaldehyde (0.1 mM) had a similar inhibitory effect on the oxidation of phenanthridine, *N*-methylphthalazinium (Table 5) and 2-hydroxybenzaldehyde

hyde, noradrenaline and dopamine not only inhibit aldehyde oxidase, but also the oxidation of charged and uncharged heterocycles. This assumption is also supported by the inhibition of phthalazine and 4-hydroxy-3-methoxybenzaldehyde (vanillin) oxidation by aldehyde oxidase, monitored by HPLC (Panoutsopoulos, 1994).

Kinetic constants for the oxidation of substituted benzaldehydes with bovine milk xanthine oxidase

In this study, all the compounds examined were found to be substrates for bovine milk xanthine oxidase. K_m values for these benzaldehydes were at least ten-fold higher than with aldehyde oxidase, but with the exception of 3,4-dihydroxybenzaldehyde, they did not vary significantly (Table 6). The lowest K_m value was obtained with 3,4-dihydroxybenzaldehyde but this was still 5–10 fold higher than that of xanthine (K_m= 0.004–0.008 mM) (Krenitsky *et al.*, 1986; Spector *et al.*, 1986).

Oxidation rates varied to a greater extent between the compounds examined with the highest V_{max} value being that of 4-hydroxybenzaldehyde which was thus the best substrate in term of substrate efficiency (K_s). The monosubstituted hydroxy-benzaldehydes gave higher V_{max} values than the analogous monosubstituted methoxy-derivatives.

Whilst 3,4-dihydroxy-, and 3-hydroxy-4-methoxy-benzaldehydes are potent inhibitors of aldehyde oxidase *in vitro*, they do not appear to exert a similar inhibitory effect upon xanthine oxidase. Therefore, it would appear that the presence of a 3-hydroxy group did not hinder the oxidation reaction catalysed by bovine milk xanthine oxidase.

Krenitsky *et al.* (1986) found benzaldehyde to be a relatively poor substrate for human liver xanthine oxidase with a low affinity for the enzyme (K_m = 0.93 mM). This value was in a similar range to the one reported in the present studies using bovine milk xanthine oxidase.

Oxidation of 2-hydroxybenzaldehyde has also been reported by Morpeth (1983) (K_m= 1 mM) for bovine milk xanthine oxidase. This K_m value is five-fold higher than that reported in this study. However, this discrepancy is probably due to the different experimental conditions used. In addition, Morpeth (1983) found the K_m for 2,5-dihydroxybenzaldehyde with bovine milk xanthine oxidase to be 0.068 mM, whereas Xia *et al.* (1999) found this substrate to have a K_m value of 0.004 mM at pH 7.0.

Purines, which are excellent substrates for xanthine oxidase, have K_m values in the order of 10^{-4} to 10^{-3} mM (Krenitsky *et al.*, 1986). Oxidation rates of substituted purines are also significantly higher than those of benzaldehydes making the latter group of compounds relatively weak substrates. Although some of these compounds were transformed as rapidly as xanthine their high K_m values make them relatively inefficient substrates of xanthine oxidase.

Inhibitors of xanthine oxidase

Noradrenaline and dopamine were also tested as inhibitors of xanthine oxidase. In contrast to the results obtained with aldehyde oxidase, they were found to be very weak inhibitors of benzaldehyde oxidation catalysed by xanthine oxidase (Table 7).

Table 7. The inhibitory effect of amines on benzaldehyde oxidation by bovine milk xanthine oxidase.

Inhibitors	Inhibitor concentration (mM)	Inhibition of benzaldehyde oxidation* (%)
Noradrenaline	1 0.1	30 15
Dopamine	1 0.1	23 23

*Concentration of benzaldehyde = 0.1 mM

CONCLUSIONS

The two enzymes used for the spectrophotometric determination of the kinetic constants were guinea pig liver aldehyde oxidase and bovine milk xanthine oxidase. These two enzymes were chosen as they have similar substrate specificity to human liver aldehyde (Beedham *et al.*, 1987a; Beedham *et al.*, 1990) and xanthine (Krenitsky *et al.*, 1986) oxidases.

The kinetic constants have indicated that the hydroxy-, and methoxy-benzaldehydes are excellent substrates for aldehyde oxidase but less efficient substrates for xanthine oxidase. Although the oxidation rates for some of the compounds with xanthine oxidase are quite comparable to those for xanthine, the high K_m values make these compounds relatively poor substrates for the enzyme.

From these studies it appears, therefore, that the activity of aldehyde oxidase may be a significant factor in the oxidation of aromatic aldehydes either generated from biogenic amines, or present in foods. They occur as natural (flavouring) constituents in a variety of foods and food components and are also widely used as food additives (Coughlan, 1980; Feron *et al.*, 1991).

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