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1-SUBSTITUTED PHTHALAZINES AS PROBES OF THE SUBSTRATE-BINDING SITE OF MAMMALIAN MOLYBDENUM HYDROXYLASES

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Abstract—The interaction of a series of 1-substituted phthalazine derivatives with partially purified aldehyde oxidase from rabbit, guinea-pig and baboon liver, and with bovine milk xanthine oxidase, has been investigated. Of the 18 compounds examined, rabbit liver aldehyde oxidase metabolized 10, whereas guinea-pig and baboon liver enzyme oxidized 13 and 14, respectively. Where metabolites were characterized, oxidation was shown to occur at position four of the phthalazine ring. K_m values ranged from 0.003 to 1.8 mM. In contrast, most compounds were competitive inhibitors of bovine milk xanthine oxidase with K_i values ranging from 0.015 to 1.3 mM; the cationic derivative 2-methylphthalazinium iodide was oxidized to 2-methyl-1-phthalazinone by both aldehyde oxidase and, with a much reduced affinity, by xanthine oxidase. In terms of structure–metabolism relationships, V_{max} values were relatively insensitive to the electronic effects of substituents, but a trend for the more lipophilic derivatives to show increased affinities (K_m and V_{max}/K_m) towards aldehyde oxidase could be seen. However, calculations of molecular size revealed a species-dependent cut-off threshold above which compounds were not metabolized. Results suggest that the relative size of the active site for hepatic aldehyde oxidase is in the order baboon > guinea-pig > rabbit, and that in spatial terms the active site of bovine milk xanthine oxidase is similar to that of baboon liver aldehyde oxidase. Thus, the binding site of rabbit liver aldehyde oxidase, a widely used source of the oxidase, is apparently more restricted than in some other species.

We have recently reported that there is a marked species variation in the *in vitro* interaction of N-heterocyclic substrates with hepatic aldehyde oxidase (EC.1.2.3.1), an oxidative enzyme of the molybdenum hydroxylase family [1]. This is true particularly for compounds based on the phthalazine (**1**) nucleus; Phthalazine itself is rapidly oxidized by hepatic aldehyde oxidase from most species examined including baboon, guinea-pig, rabbit and man. In contrast, the substituted phthalazine, carbazeran (**10**) has a high affinity for enzyme from baboon, guinea-pig or human liver but is refractory to oxidation by rabbit liver aldehyde oxidase. Carbazeran undergoes complete clearance presystemically in man and baboon via 4-hydroxylation of the phthalazine moiety, a reaction shown to be catalysed by hepatic aldehyde oxidase [2, 3].

Aldehyde oxidase has traditionally been prepared from rabbit liver. The lapine enzyme appears to have a wide substrate specificity and undoubtedly displays a high activity with some frequently-used substrates [4, 5]. However, it is now apparent that rabbit enzyme is not always representative of aldehyde oxidase activity from other mammalian species including man [5, 6]. In contrast to rabbit, relatively little is known about baboon and guinea-pig liver aldehyde oxidase, but both appear to have a closer spectrum of activity to human liver enzyme [1]. The present studies were undertaken to compare the substrate/inhibitor specificities of rabbit, guinea-pig and baboon liver aldehyde oxidase towards various substituted phthalazines.

In addition, the activity of another molybdenum hydroxylase namely bovine milk xanthine oxidase (EC 1.2.3.2) towards these compounds was investigated. The latter enzyme, also found in liver, has an overlapping, complementary substrate specificity to the closely related aldehyde oxidase [7]. For example, xanthine oxidase also catalyses the oxidation of phthalazine, albeit less efficiently than aldehyde oxidase [8]. It has thus been suggested that aldehyde oxidase and xanthine oxidase provide a protective barrier for the detoxification of ingested nitrogen-containing heterocycles since high concentrations of these enzymes are found in the liver (both molybdenum hydroxylases) and small intestine (xanthine oxidase) [6, 7].

MATERIALS AND METHODS

Chemicals. Most of the substituted phthalazines were donated from the Compound Control Centre of Pfizer Central Research (Sandwich, U.K.) having been synthesized according to published procedures. 1-Phthalazinone [9], 1-chlorophthalazine [10], 2-methylphthalazinium iodide [11] and 2-methyl-1-phthalazinone [12] were synthesized by literature methods; phthalazine was purchased from the Aldrich Chemical Co. (Gillingham, U.K.). All the substituted phthalazines were characterized by melting point, i.r. spectroscopy and mass spectrometry. Structures of the substituted phthalazines are shown

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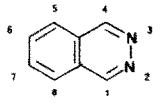
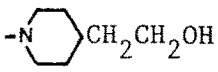
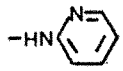
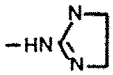
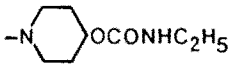
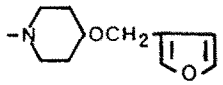
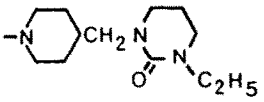
Table 1. Structures of 1-substituted phthalazines



Compound no.	Substituent R_1	R_5	R_6	R_7
1	H	H	H	H
2	Cl	H	H	H
3	Cl	H	OCH_3	OCH_3
4	OC_2H_5	H	H	H
5	C_6H_5	H	H	H
6	OC_6H_5	H	H	H
7		H	H	H
8		H	H	H
9		H	H	H
10		H	OCH_3	OCH_3
11		OCH_3	OCH_3	OCH_3
12		H	OCH_3	OCH_3
13		H	OCH_3	OCH_3
14	—$NHCH_2$ CH_2—$N(CH_3)_2$	H	H	H

in Table 1. The solvents used for high-performance liquid chromatography (HPLC) were of HPLC grade. All other chemicals were of reagent grade.

Enzyme purification. Partially purified aldehyde oxidase was prepared by the procedure of Johnson *et al.* [13] from freshly excised livers of mature, male New Zealand White rabbits or Dunkin–Hartley guinea pigs killed by cervical dislocation at approximately 9:00 a.m. Frozen baboon liver, supplied by Pfizer Central Research, was processed in a similar manner. Enzyme fractions were stored as pellets in liquid N_2, with activity remaining constant for 3–6 months.

Bovine xanthine oxidase (Grade I) from buttermilk was purchased as a suspension from the Sigma Chemical Co. (Poole, U.K.) and stored at 4°.

Spectrophotometric measurement of substrate oxidation. Unless otherwise specified, oxidation rates with the molybdenum hydroxylases were measured at 37° as previously described [13]. Assay mixtures contained varying concentrations of substrate, potassium ferricyanide ($K_3Fe(CN)_6$) (1 mM), EDTA (0.13 mM) in a final volume of 3 mL potassium phosphate buffer, pH 7 (67 mM) and sufficient enzyme to produce measurable rates over the range of substrate concentrations under study (usually 100 μL of appropriately diluted enzyme). Reduction of the electron acceptor was monitored at 420 nm and all compounds were tested for non-enzymic reduction or for interaction with $K_3Fe(CN)_6$.

V_{max} and K_m values were determined by measuring initial oxidation rates at a minimum of seven different

substrate concentrations for a range of concentrations bracketing the K_m value. Kinetic constants were calculated by computer using a Lineweaver–Burk plot and expressing the goodness of fit of the line by the 'coefficient of determination'.

Oxidation of 2-methylphthalazinium iodide was also monitored in the absence of $K_3Fe(CN)_6$ by monitoring an increase in absorbance at 315 nm. No significant difference was found for the oxidation rate of 2-methylphthalazinium iodide when either O_2 or $K_3Fe(CN)_6$ functioned as the electron acceptor.

Compounds not exhibiting substrate activity were tested as inhibitors of the oxidation of phthalazine by aldehyde oxidase preparations and commercial xanthine oxidase. Owing to the limited availability of the substituted phthalazines, oxidation rates for at least seven concentrations of the variable substrate, phthalazine, were determined in the presence of a single concentration of inhibitor and compared to uninhibited reaction rates. With the exception of dihydralazine, inhibition was competitive and the inhibition constant was calculated from Lineweaver–Burk plots using the following formula:

$$K_{m^i} = K_m \left(1 + \frac{[I]}{K_i}\right).$$

Dihydralazine was found to interact with the electron acceptor, $K_3Fe(CN)_6$. Thus $K_3Fe(CN)_6$ was omitted from incubation mixtures, and initial oxidation rates of methotrexate (50 μM), purine (0.8 mM), xanthine (10 μM) or *N*-methylphthalazinium iodide (0.1–10 mM) were monitored directly at 340, 285, 295 and 315 nm, respectively, in the presence of varying concentrations of dihydralazine. In such cases, molecular O_2 acts as the electron acceptor.

Identification of in vitro *oxidation products. In vitro* oxidation products were isolated using one of the following methods:

(i) Partially purified guinea-pig or rabbit aldehyde oxidase (2 mL) was added in aliquots of 0.5 mL every 30 min to an incubation mixture (20 mL) containing 1 mM substrate and 0.1 mM EDTA in 67 mM potassium phosphate buffer pH 7, agitated in a shaking water bath at 37°. Controls omitting enzyme were incubated in parallel. The reaction was terminated after 2 hr by the addition of solid Na_2SO_4 followed by heating on a steam bath for 10 min. The protein precipitate was removed by centrifugation, the product extracted with ethyl acetate (10 mL + 2 × 5 mL) which was evaporated to dryness under nitrogen and the residue dissolved in methanol (100 μL).

(ii) The compounds were incubated in a volume of 10 mL as (i) for 5 hr and the reaction terminated by the addition of 5 mL ice-cold, 20% (w/v) aqueous trichloroacetic acid. After removal of the protein precipitate by centrifugation, the supernatant was passed down a CN-Bond Elut solid phase extraction column (Analytichem International) and the products eluted with methanol. The methanol extract was concentrated under nitrogen to a volume of 100 μL.

Chromatography. HPLC analysis was performed at ambient temperature using a Waters 501 pump, U6K injection loop and a Lambda max model 481 LC variable wavelength detector connected to a 740 data module. Substrate and products were separated on reverse phase columns (20 cm × 4 mm i.d.) packed with CN Spherisorb or CN CPS Hypersil (5 μM particle size) with 1% (w/v) ammonium acetate/methanol (3:1, v/v) as the mobile phase. The eluent was monitored at 270 or 285 nm and the flow rate was 1 mL/min. Aliquots (25 μL) of the methanol concentrate were injected on to the HPLC system and portions of the eluate corresponding to each substrate and metabolite were collected separately. Fractions from successive injections were combined, the methanol removed under nitrogen at 37° and the aqueous component freeze-dried overnight. The residue was analysed by i.r. spectroscopy and mass spectrometry as previously described [14].

Determination of log K_o *values.* The assessment of relative lipophilicities by reverse-phase HPLC has been shown to be a convenient alternative, requiring only small samples, compared to the time consuming and compound intensive shake-flask or empirical methods [15]. A linear relationship between log K_o and log P holds for many groups of compounds.

Capacity factors (K') of the substituted phthalazines were determined by reverse-phase HPLC with either (i) a Waters Baseline 810 Data and Control Station equipped with two 510 pumps, a WISP 712 automatic injection system and a Model 455 variable wavelength detector or (ii), a Perkin–Elmer Series 410 pump and ISS.100 Auto-injector connected to a Kratos Spectroflow 773 detector and Spectra-Physics SP4-270 integrator.

A Spherisorb S5 ODS 2 column (12.5 cm × 4.9 mm) was employed at ambient temperature with at least three different mobile phases comprising of varying concentrations of methanol in 10 mM *N,N,N,N*-tetramethylethylenediamine (TEMED) buffer pH 7.4. The mobile phase was monitored at 270 nm with a flow rate of 1 mL/min.

Capacity factors were calculated as follows:

$$K = \frac{(t_R - t_o)}{t_o}$$

where t_R and t_o are the retention times on the column of the compound and a non-retained peak, respectively. Log K was linearly regressed against % methanol in the mobile phase; and the graphs extrapolated to give a theoretical log K at 0% methanol, this being denoted log K_o.

RESULTS

Identification of metabolic oxidation products

Chromatographic and spectral characteristics of the metabolites isolated from incubations of compounds **4–8** with guinea-pig or rabbit liver enzyme are shown in Table 2. Mass spectra of all metabolites displayed an ion that was 16 a.m.u. higher than the corresponding substrate indicating the incorporation of one oxygen atom into the parent nucleus. In addition, a strong peak around 1640 cm^{-1} characteristic of a carbonyl group in a conjugated amide was present in the i.r. spectra of all oxidation products. Consequently, oxidation is presumed to have occurred in the heterocyclic ring at carbon 4 as this

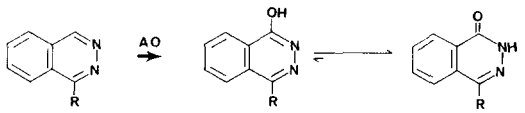
Table 2. Chromatographic and spectral analysis of aldehyde oxidase-catalysed oxidation products

Compound	HPLC R_V (mL)	i.r. spectra: υ(CO)(cm^{-1})	Mass spectra: (m/z)
1-Ethoxyphthalazine	13.5	—*	174,146†
Enzymic oxidation product	9.10	1660	190,162
1-Phenylphthalazine	14.18	—	206,205
1-Phenyl-4-phthalazinone	9.80	1660	222,221,165
Enzymic oxidation product	9.82	1650	222,221,165
1-Phenoxyphthalazine	15.5	—	222,221,91
Enzymic oxidation product	11.5	1650	238,91
7	19.2	—	257,36
Enzymic oxidation product	9.5	1625	273,82
8	15.0	—	222,221,78
Enzymic oxidation product	13.0	1640	238,237,78
2-Methylphthalazinum iodide	—	—	145,143
2-Methyl-1-phthalazinone	12.2	1630	160,132
Enzymic oxidation product	12.2	1630	160,132
9		—	212,159,141
Enzymic oxidation product		1660	228,160,159

* No peak.

† Values underlined represent the most abundant ion.

is the only hydroxylated metabolite that exists as a lactam tautomer (Scheme 1).



Scheme 1.

Interaction of 1-substituted phthalazines with liver aldehyde oxidase

The kinetic constants, K_m and V_{max} for the oxidation of phthalazines are presented in Table 3. As supplies of most of the substituted phthalazines were limiting it was not possible to make more than one determination of a K_m value for each compound with liver from each species. However, the standard errors calculated for the K_m value of carbazeran (**10**), for five different hepatic guinea-pig preparations was only ±1.2% while that for the V_{max} value was ±9%. These are comparable with those quoted in other studies [16] and are taken as an indication of intra-species variation in the kinetics for this group of compounds.

Enzyme from all three species showed the highest V_{max} values with unsubstituted phthalazine (**1**). In electronic terms, initial attack by both aldehyde oxidase and xanthine oxidase is nucleophilic occurring at an electron-deficient carbon. However, despite the net electron-withdrawing effect of a chloro group, the enzymic oxidation rate for 1-chlorophthalazine (**2**) is, in each case, less than that observed with phthalazine, although the overall efficiency (V_{max}/K_m) of rabbit liver aldehyde oxidase towards this compound is significantly increased (see Fig. 1). A similar effect has been noted for 6-substituted purines where strongly electron-withdrawing groups increase substrate efficiency towards rabbit liver enzyme although V_{max} values of such compounds were found to be less than that of purine itself [17].

1-Phenylphthalazine (**5**), which is the most lipophilic compound studied (Table 4), has the lowest K_m values with both baboon and guinea-pig liver enzyme and is a good substrate of rabbit liver aldehyde oxidase (Table 3). A trend for the more lipophilic phthalazines to show increased affinities towards aldehyde oxidase can be seen.

The interspecies variation in the V_{max} values for 1-[4-(hydroxyethyl)piperidino]phthalazine (**7**) is significantly greater than that observed for compounds **1–6**. Thus the oxidation of **7** by rabbit liver aldehyde oxidase is almost completely abolished by the substituent although a low K_m value is obtained. A similar but less pronounced effect is observed with guinea-pig enzyme whereas baboon aldehyde oxidase gives a relatively high V_{max} combined with a high K_m value. Species differences between the binding sites of aldehyde oxidase are even more apparent when there are additional substituents in the phthalazine molecule. Thus, the other 1,6,7-trisubstituted-(**10**, **12**, **13**) and 1,5,6,7-tetrasubstituted-phthalazines (**11**) do not bind to rabbit liver aldehyde oxidase either as substrates or inhibitors of phthalazine oxidation. Carbazeran (**10**) has a high affinity for both guinea-pig and baboon liver enzyme. However, an additional methoxy substituent at carbon 5 (**11**) decreased substrate efficiency markedly for guinea pig liver enzyme with less effect on baboon aldehyde oxidase. The latter is the only enzyme able to react with compound **13** which has an extremely bulky substituent at carbon 1.

Calculated molar refractivities (CMR) are crude but useful measures of the overall size of each molecule [18]. The variation in CMR for these phthalazines with efficiency (V_{max}/K_m) of aldehyde oxidase catalysed oxidation for each species is illustrated in Fig. 1.

Table 3. Kinetic constants for oxidation of phthalazines by liver aldehyde oxidase

Compound no.	K_m† (mM)			V_{max} (μmol/min/mg)		
	R	G	B	R	G	B
1	0.110	0.052	0.014	0.727	0.542	0.697
2	0.036	0.039	0.030	0.512	0.413	0.115
3	0.039	0.045	0.011	0.095	0.177	0.048
4	0.218	0.051	0.013	0.094	0.090	0.088
5	0.201	0.029	<0.003	0.155	0.087	0.047
6	0.355	0.103	0.029	0.006	0.003	0.009
7	0.072	0.044	0.120	0.0003	0.026	0.137
8	0.280	0.580	0.340	0.021	0.003	0.091
9	1.800	high	1.100	0.008	0.002	0.047
10	*	0.053	0.009	0	0.030	0.067
11	—	0.480	0.048	0	0.005	0.013
12	—	0.074	0.016	0	0.034	0.035
13	—	—	0.93	0	—	0.014

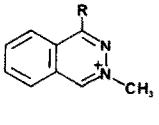
Kinetic constants were determined at pH 7 and 37°.
Key to species: R, rabbit; G, guinea-pig; B, baboon.
* No interaction.
† Mean correlation coefficient for K_m determinations $c = 0.998$ (see experimental methods).

Interaction of 1-substituted phthalazines with bovine milk xanthine oxidase

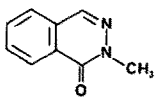
With the exception of **2** and **7**, 1-substituted phthalazines were not oxidized by bovine milk xanthine oxidase. Substitution of a chlorine atom did not alter the K_m or V_{max} values markedly (V_{max} for 1-chlorophthalazine = 0.128 μmol/min/mg) but the more lipophilic 4-hydroxyethylpiperidino-group appeared to enhance binding of the substrate (K_m decreased by 80%) but to hinder the oxidation (V_{max} for **7** = 0.006 μmol/min/mg).

The remaining substituted phthalazines were moderate competitive inhibitors of the enzyme with K_i values ranging from 0.015 to 1.3 mM (Table 5). A typical example is presented in Fig. 2. To put these data into perspective, extensive studies have previously identified inhibitors, both competitive and non-competitive, with I_{50} values in the micro and sub micro-molar range (see Ref. 6 for review).

N-Methylphthalazinium iodide as a substrate of the molybdenum hydroxylases



15a R = H
15b R = O^-



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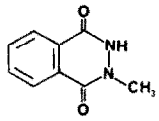

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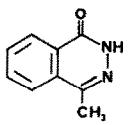
There were only minor species differences between the kinetic constants for the reaction of the cationic substrate, 2-methylphthlazinium iodide (**15a**) with hepatic aldehyde oxidase (Table 6). Despite the increased susceptibility of quaternary compounds to nucleophilic oxidation, the K_m value, in each species, was higher than its unquaternized counterpart (phthalazine) and V_{max} values were depressed. Enzymic oxidation of **15a**, like that of phthalazine, generates only one metabolite although there are two potential positions (carbons 1 and 4) for aldehyde oxidase attack to give either **16** or **15b** together with the possibility of a dioxygenated product (**17**).

The compound isolated from the reaction showed identical TLC and HPLC retention values to authentic 2-methyl-1-(2H)-phthalazinone (**16**) (Table 6). In addition, the i.r. spectrum of the metabolite contained a strong band at 1630 cm^{-1} indicative of a lactam tautomer, whereas the C—O band in the i.r. spectrum of the betaine (**15b**) occurs at 1550 cm^{-1} [9]. The identity of the oxidation product was confirmed as **16** from the mass spectrum which exhibited a molecular ion peak at 15 a.m.u. greater than that of the substrate (Table 2). Thus 2-methyl-1-phthalazinone is not subject to further oxidation at carbon 4. In fact, this compound is a competitive inhibitor of aldehyde oxidase from each of the three species (Table 6). Similarly, 1-phthalazinone has previously been shown to be a weak competitive inhibitor of rabbit liver aldehyde oxidase [19]. 1-Methyl-4-phthalazinone (**18**) which lacks a suitable site for oxidation also gave K_i values of 1.8, 0.29 and 0.48 mM with rabbit, guinea-pig and baboon liver enzyme, respectively.



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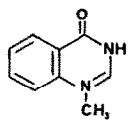

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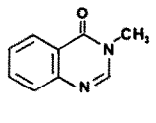

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Although a number of heteroaromatic cations are readily oxidized by bovine milk xanthine oxidase in basic solutions, such compounds do not show appreciable oxidation rates at physiological pH values [20]. In the present study, *N*-methylphthalazinium iodide (**15a**) was also a substrate of

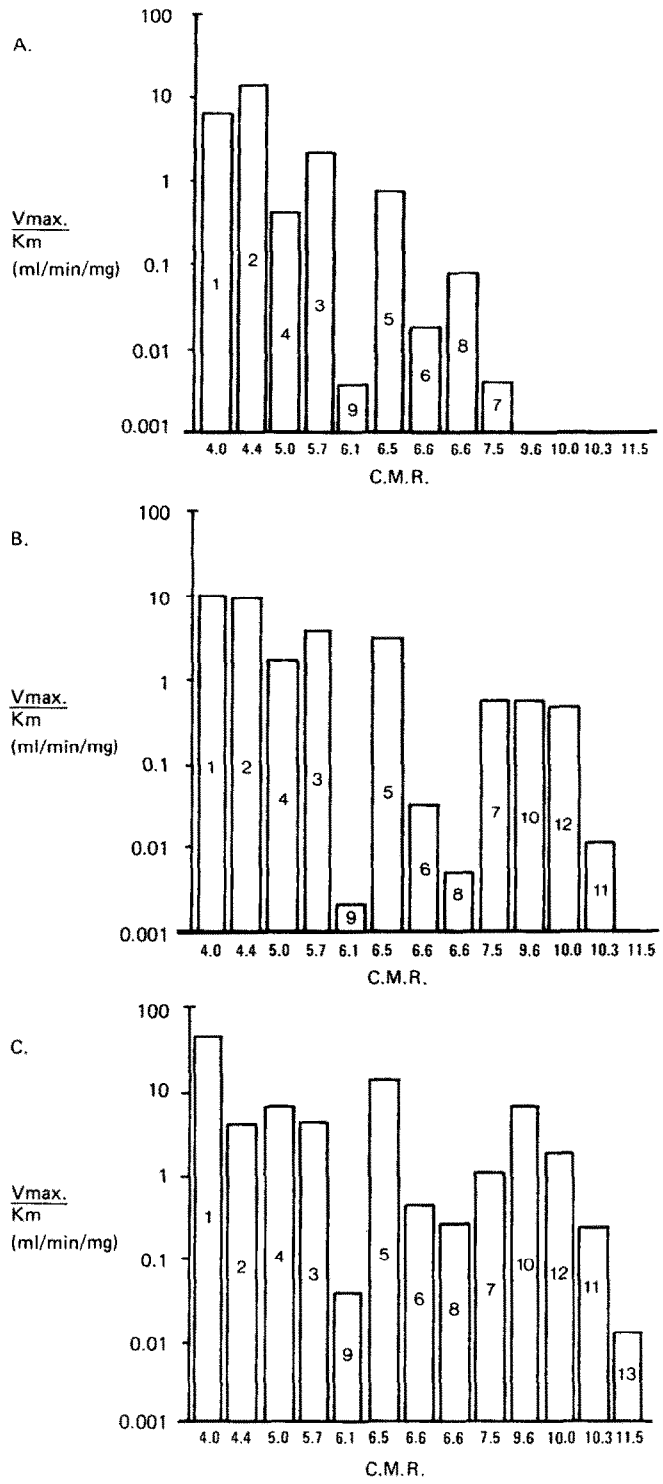

Fig. 1. Relationship between calculated molar refractivity (CMR) and V_{max}/K_m values for aldehyde oxidase mediated metabolism of phthalazine derivatives. (A) Rabbit; (B) guinea-pig; (C) baboon. Compound numbers indicated on bars.

bovine milk xanthine oxidase (Table 6) but with a much decreased affinity than for aldehyde oxidase. The K_m value for **15a** was similar to that of phthalazine (K_m = 2 mM). However the oxidation rate and hence V_{max}/K_m were substantially decreased (V_{max} for phthalazine = 0.135 μmol/min/mg). As with aldehyde oxidase, 2-methyl-1-phthalazinone (**16**) (Table 6) and 1-methyl-4-phthalazinone (**18**) (Table 5) were both competitive inhibitors of bovine milk xanthine oxidase. This is in contrast to the isomeric

Table 4. Relative lipophilicities and calculated molar refractivities of substituted phthalazines

Compound no.	log K_o	CMR	Compound no.	log K_o	CMR
1	0.5	3.954	**8**	3.0	6.623
2	0.3	4.446	**9**	0.2	6.097
3	1.5	5.680	**10**	1.8	9.647
4	ND	5.035	**11**	ND	10.264
5	3.9	6.466	**12**	3.2	10.041
6	1.9	6.619	**13**	ND	11.541
7	1.6	7.545			

Capacity factors (K') were determined by reverse-phase HPLC as described in Materials and Methods and log K_o calculated from plots of log K' versus % methanol in mobile phase.

Linear regression gave correlation coefficients ($r > 0.995$).

Calculated molar refractivities (CMR) were determined using the MEDCHEM computer program (Medicinal Chemistry Project, Pomona College, Claremont, CA).

ND, not determined.

Table 5. Inhibitor constants for substituted phthalazines with bovine milk xanthine oxidase

Compound no.	K_i (K_m)* (mM)	Compound no.	K_i (mM)
2	(2.4)	**9**	0.099
3	0.051	**10**	0.015
4	1.30	**11**	0.031
5	0.16	**12**	0.077
6	0.071	**13**	0.018
7	(0.4)	**14**	0.140
8	0.403	**18**	1.10
Dihydralazine	8.80		

Reaction rates in the presence of inhibitor were determined at pH 7 and 37° using phthalazine or, in the case of dihydralazine, *N*-methylphthalazinium iodide as substrates and the K_i values calculated as described in Materials and Methods.

* Values in parentheses are K_m values.

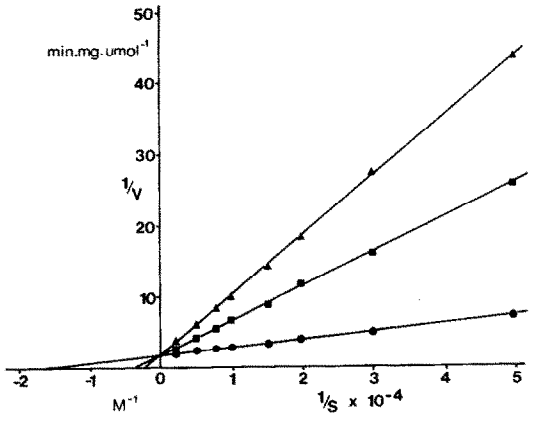

Fig. 2. Lineweaver–Burk plots for the oxidation of phthalazine by bovine milk xanthine oxidase at pH 7 and 37° in the presence of 2-methyl-1-phthalazinone (I) [●, (I) = 0; ■, (I) = 3 mM; ▲ (I) = 6 mM].

1-methyl-4-quinazolinone (**19**) which Bunting *et al.* [21] have shown to be oxidized to the corresponding dione at pH 7. However, 3-methyl-4-quinazolinone (**20**), like **16** is a competitive inhibitor of the enzyme.

Interaction of dihydralazine with molybdenum hydroxylases

We have previously demonstrated that the antihypertensive agent hydralazine (1-hydrazinophthalazine) is a potent inhibitor of rabbit, guinea-pig or baboon liver aldehyde oxidase both *in vitro* and *in vivo* [22]. The 1,4-dihydrazino-analogue (dihydralazine) in the present study showed similar inhibition characteristics being a potent progressive inhibitor of aldehyde oxidase (Fig. 3); marked inhibition was observed even at concentrations of 10 nM. However, unlike hydralazine, the *initial* oxidation rate was decreased for both rabbit and guinea-pig liver enzyme; baboon liver was not tested in the present study. Pre-incubation of dihydralazine with aldehyde oxidase for 30 min gave the same inhibited progress curve to that when the compound was added immediately prior to substrate.

Dihydralazine was found to be a weak competitive inhibitor of bovine milk xanthine oxidase (Table 6) whereas no reaction had been observed for hydralazine [22]. However, it is possible that hydralazine would also inhibit xanthine oxidase if tested at comparable concentrations.

DISCUSSION

In order to determine which physical properties of the substituted phthalazines may be important in governing substrate activity towards aldehyde oxidase it had to be established that the position of oxidation occurred at an equivalent carbon in each compound.

Phthalazine (**1**) has been previously shown to undergo oxidation by aldehyde oxidase at carbon 1 [8] and 1-chlorophthalazine (**2**) is converted to 1-chloro-4-phthalazinone [13]. Products isolated from incubations of the other 1-substituted phthalazines

Table 6. Kinetic constants for molybdenum hydroxylase catalysed oxidation of 2-methylphthalazinium iodide

Kinetic constant	Rabbit liver aldehyde oxidase	Guinea-pig liver aldehyde oxidase	Baboon liver aldehyde oxidase	Bovine milk xanthine oxidase
K_m (mM)	0.18	0.14	0.16	2.30
V_{max} (μmol/min/mg)	0.314	0.182	0.507	0.010
V_{max}/K_m (ml/min/mg)	1.744	1.3	3.17	0.004
K_i^* (mM)	0.31	0.94	0.084	0.98

* K_i of 2-methyl-1-phthalazinone (**16**) as a competitive inhibitor of phthalazine oxidation.

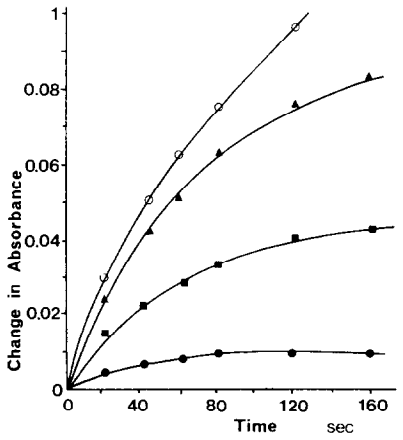

Fig. 3. Dihydralazine inhibition of purine (0.8 mM) oxidation catalysed by rabbit liver aldehyde oxidase at pH 7 and 37°. Concentration of dihydralazine (○) 0; (▲) 0.001 μM; (■) 1 μM; (●) 0.1 mM.

(compounds **4**–**9**) in the present study had also undergone oxidation at carbon 4 in the phthalazine ring (see Scheme 1). 1-(2-Pyridino)aminopthalazine (**8**) contains a second heteroaromatic ring, which if oxidized by aldehyde oxidase would also produce a cyclic lactam. However, the mass spectra of **8** and its oxidation product both included a strong peak at 78 a.m.u. corresponding to a pyridine ring and not a pyridone fragment. Carbazeran (**1**), a 1,6,7-trisubstituted phthalazine, is converted to a 4-oxo metabolite by baboon and rabbit liver aldehyde oxidase [2, 3] thus it is likely that similar compounds (**11**–**13**) are also oxidized at carbon 4 by the enzyme.

It appears that for compounds based on the phthalazine nucleus the rate of oxidation is not very sensitive to the electronic effects of substituents. Therefore it is not possible to construct a linear Hammett plot of log(relative maximal oxidation rates) versus the substituent constant with the results from this study. This is in contrast to cationic substrates of rabbit liver aldehyde oxidase where the reaction is facilitated by a low electron density at the reaction site [23]. The decreased sensitivity to substituent effects in uncharged heterocycles may be a further indication of a different rate-limiting step in enzymic oxidation to that of cationic substrates. Further investigations into the role of electron density in determining substrate reactivity for substituted phthalazines and related series are in progress.

Lipophilicity is a second important parameter in determining structure–activity relationships. In this study log K_0 values of the substituted phthalazines were determined by reverse-phase liquid chromatography. However, there was no simple relationship between lipophilicity of these compounds and binding to enzyme from any species. Hydrophobic interaction in the binding site of rabbit liver aldehyde oxidase has been shown to facilitate binding of other diazanaphthalenes [8, 24] and substituted purines [17], although the positioning of hydrophobic groups is critical in governing substrate activity.

The present investigation has re-emphasized the potential of mammalian liver aldehyde oxidase to metabolize N-heterocycles. However, despite the wide substrate specificity of rabbit liver enzyme demonstrated in this and previous studies, it is now apparent that the binding site of this enzyme is more restricted than in other species, with that of baboon being able to accommodate the most bulky substrates. This is clearly illustrated in Fig. 1 which shows that there are no substituted phthalazines with CMR values greater than 8 that bind to rabbit liver aldehyde oxidase whereas the limit for binding to the guinea-pig enzyme appears to be a CMR value of around 10. Thus, spatial restrictions in the binding site of guinea-pig liver aldehyde oxidase seem to lie somewhere between those of rabbit and baboon. We have previously shown that aldehyde oxidase from guinea-pig liver resembles that of man in its *in vitro* activity towards phthalazines and quaternary substrates [1], and the interaction of human hepatic aldehyde oxidase with these substituted phthalazines and other N-heterocycles is currently being investigated in our laboratories.

As those compounds showing the most variation in activity between species all have a nitrogen atom attached directly to the phthalazine ring at position 1, there is also a possibility that the pK_a of these compounds may account for the observed interspecies differences. However, this is not supported by the limited pK_a data for phthalazines shown below:

	pK_a
Phthalazine (**1**)	3.47
1-Aminophthalazine	6.57
Carbazeran (**10**)	5.19.

Compound 7, which is less bulky than carbazeran, also has a piperidine ring at position 1 of the phthalazine nucleus and thus will probably have a similar pK_a. However, carbazeran did not react with the lapine enzyme whereas compound 7 is a substrate. Furthermore, 1-aminophthalazine is also a substrate for rabbit liver aldehyde oxidase at pH 7.0 [13] whereas carbazeran does not react although there is little difference in the pK_a values for these compounds.

As most of the substituted phthalazines competitively inhibited the oxidation of phthalazine by xanthine oxidase it is likely that they are binding at the same site as the substrate. Consequently it would appear, on comparison of Tables 3 and 5, that bovine milk xanthine oxidase has a much larger active site than rabbit or guinea-pig liver aldehyde oxidase but that the spatial restrictions within the site are probably similar to those in baboon liver aldehyde oxidase. Binding to xanthine oxidase has been shown to be assisted by hydrophobic substituents although the position of the group within the molecule is critical [6]. This trend is also apparent in the present study since 1-phenylphthalazine binds more strongly to the enzyme than phthalazine but other less lipophilic compounds are more potent inhibitors. Electronegative atoms (e.g. N, O) in substituent groups are also thought to be important in the binding of substrates to xanthine oxidase [25]. The greater affinity of the 1-substituted phthalazines may be a combination of increased lipophilicity and additional electronegative atoms which enhance non-productive binding modes in a similar way to that proposed for substrates.

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