Biochemical Pharmacology, Vol. 34, No. 24, pp. 4251–4256, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

HYDRALAZINE: A POTENT INHIBITOR OF ALDEHYDE OXIDASE ACTIVITY *IN VITRO* AND *IN VIVO*

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(*Received* 18 *March* 1985; *accepted* 8 *July* 1985)

Abstract—The interaction of the vasodilator, hydralazine, with the molybdenum hydroxylases, aldehyde oxidase and xanthine oxidase has been investigated. A potent progressive inhibition of rabbit liver aldehyde oxidase, in the presence of substrate, by low concentrations of hydralazine (0.1–1 μM) was observed *in vitro* but no effect was seen with bovine milk xanthine oxidase. This activity was mirrored *in vivo* when levels of aldehyde oxidase were significantly decreased in rabbits administered hydralazine (10 mg/kg/day for seven days) whereas hepatic xanthine oxidase activity was unaltered by hydralazine treatment. Various metabolites of hydralazine were synthesized but found to be devoid of *in vitro* inhibitory activity. Aldehyde oxidase prepared from either guinea pig or baboon liver was inhibited in a similar way to that of rabbit liver.

Administration of phthalazine (2,3-diazanapthalene) or its enzymic oxidation product, 1-phthalazinone, to rabbits increases the activity of both hepatic molybdenum hydroxylases, aldehyde oxidase (aldehyde:O_2 oxidoreductase EC 1.2.3.1) and xanthine oxidase (xanthine:O_2 oxidoreductase EC 1.2.3.2 [1]. Phthalazine is a substrate for both these molybdenum-containing enzymes although it is oxidized much more rapidly by the former [2]. In contrast, 1-phthalazinone is a weak inhibitor of both enzymes [1].

Hydralazine, i.e. 1-hydrazinophthalazine, is a widely used anti-hypertensive agent which is extensively metabolized in a number of species, including man [3–5] and rabbit [6, 7]. Phenolic microsomal products have been isolated from rats and rabbits [6, 8, 9] but in man two major pathways account for most of the metabolites [3, 4]; *N*-acetylation of the hydralazine moeity followed by ring closure leads to a series of triazolo-phthalazines and oxidation of the heterocyclic ring gives rise to phthalazinone derivatives. The relative contribution of these two pathways is influenced by the acetylator phenotype and 1-phthalazinone is produced in larger amounts in slow acetylators [3, 4]. The metabolic origin of phthalazinone is still uncertain; both microsomal enzymes [3] and aldehyde oxidase [2] have been implicated. Nevertheless, formation of this compound from hydralazine, by whatever route, could also lead to increased levels of the molybdenum hydroxylases.

However, hydralazine itself is an effective inhibitor of a number of enzymes both *in vivo* and *in vitro* including various amine oxidase enzymes [10, 11], dopa decarboxylase [12], tyrosine hydroxylase [13] and lysyl oxidase [14]. There is also evidence that hydralazine inhibits microsomal enzymes *in vitro* but this result is not mirrored *in vivo* [15]. In a preliminary communication we have previously reported that hydralazine was a potent inhibitor of aldehyde oxidase and that the drug considerably reduced the oxidation of phthalazine by rabbit liver slices [16]. This paper describes in detail the effect of hydralazine on molybdenum hydroxylase activity both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Chemicals. Phthalazine, purine and phenanthridine were purchased from Aldrich Chemical Company (Gillingham, U.K.), 3-methylisoquinoline from ICN Pharmaceuticals Inc. (K & K) (Irvine, CA) and xanthine was obtained from Koch-Light Laboratories Ltd. (Colnbrook, U.K.). Hydralazine hydrochloride was supplied by Sigma Chemical Co. Ltd. (London, U.K), and methotrexate was a gift of the Cancer Research Unit, University of Bradford. 3-Methyl-S-triazolo(3,4-α)phthalazine was synthesized according to the method of Lesser *et al.* [17] and tetrazolo(5,1-α)phthalazine by the method of Druey and Ringier [18].

Pretreatment of rabbits and preparation of enzyme fractions. A solution of hydralazine hydrochloride (10 mg/kg/day) in 0.005 M potassium phosphate buffer pH 6 was administered orally to female white New Zealand rabbits for a total of seven days. The solution was renewed daily and then replaced by drinking water for 24 hr, after which time the animals were sacrificed. The liver was removed and soluble enzyme fractions prepared as described previously [1]. Control rabbits were treated either with water or 0.005 M potassium phosphate buffer pH 6. Protein determinations were carried out using the Bradford method [19].

Spectrophotometric assay of purified enzyme fractions. All activities were determined at 37°. The specific activities of hydralazine-treated and control enzyme fractions were determined spectrophotometrically as described previously [1] in 0.067 M

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potassium phosphate buffer pH 7 using either 1 mM phthalazine, 50 μM phenanthridine, 0.5 mM 3-methylisoquinoline or 50 μM xanthine as substrate.

Reaction of hydralazine with aldehyde oxidase and xanthine oxidase in vitro. Rabbit liver aldehyde oxidase was prepared as described in the previous section. Guinea-pig liver and baboon liver aldehyde oxidase was obtained in the same manner from Dunkin–Hartley male guinea-pigs and frozen baboon liver kindly donated by Pfizer Central Research (Sandwich, U.K.). Bovine milk xanthine oxidase (Grade 1, 0.5 units/mg protein) was purchased from Sigma Chemical Co. (London, U.K.).

Both substrate (i) and inhibitory (ii) activity was measured in 0.067 M phosphate buffer pH 6 containing 0.1 mM EDTA at 37°.

(i) 0.1 ml of purified aldehyde oxidase (ammonium sulphate fraction) or bovine milk xanthine oxidase was added to 0.1 mM hydralazine hydrochloride and the solution repeatedly scanned between 200–350 nm over a period of 10 min.

(ii) The initial rates of oxidation of 50 μM methotrexate (340 nm), 0.8 mM purine (285 nm) or 10 μM xanthine (295 nm) were monitored in the presence of varying concentrations of hydralazine hydrochloride. No interaction occurred between any of these substrates and hydralazine in the absence of enzyme.

Measurement of the molybdenum content and analytical isoelectrofocusing of rabbit liver aldehyde oxidase were carried out as described previously [1].

RESULTS AND DISCUSSION

Effect of hydralazine on molybdenum hydroxylase activity in vivo

The specific activities of the molybdenum hydroxylases prepared from livers of hydralazine-treated rabbits are compared to control values in Table 1. Johnson has shown that treatment with phosphate buffer alone for 7 days had no effect on molybdenum hydroxylase activity [20] and thus control values are from untreated rabbits. Hydralazine had little effect on xanthine oxidase activity, although a slight but not statistically significant increase was observed in the ammonium sulphate fraction. In contrast, hydralazine administration caused a decrease in aldehyde oxidase activity which was statistically significant to at least $P < 0.01$ for the ammonium sulphate and DEAE fractions and to $P > 0.02$ for the G75 fraction, using a two tailed Students *t*-test. The K_m value determined for phthalazine with partially purified aldehyde oxidase from hydralazine-treated rabbits was 57 μM (in 0.067 M phosphate buffer pH 7 at 37°). This compares to a value of 110 μM for enzyme from control rabbits [1].

The reduction in the catalytic activity of aldehyde oxidase does not necessarily reflect lower hepatic levels of the enzyme. A drop in activity could also arise from decreased recovery of the hydralazine-treated enzyme or aldehyde oxidase may still be present in a less active form. Consequently, the molybdenum content of the purified DEAE fractions corresponding to aldehyde oxidase activity were determined. Xanthine oxidase is not present in this fraction [20].

The molybdenum content of the DEAE-fractions from hydralazine-treated rabbits, measured using electrothermal atomic absorption spectroscopy, was found to be 0.43 ± 0.14 μg Mo/mg protein (no. of rabbits (N) = 5) compared to a value of 0.38 ± 0.06 μg Mo/mg protein (N = 3) for fractions from control rabbits. There is no significant difference between the Mo content of these enzyme fractions. Thus, in contrast to previous work with the parent compound phthalazine which caused a similar increase in both aldehyde oxidase activity and molybdenum concentration [1], hydralazine does not alter the molybdenum content of the DEAE fraction. This suggests that there is no difference in the recovery of control and hydralazine-treated enzyme during purification.

Table 1. Effect of hydralazine administration upon specific activities of rabbit liver molybdenum hydroxylases

Substrate/ fraction	Specific Activity at 37° and pH 7 (μmol/min/mg protein) Control N = 7	Hydralazine-treated N = 5	% Control
Ammonium Sulphate Fraction			
Phthalazine	0.59 ± 0.25†	0.17 ± 0.05	29
Phenanthridine	0.23 ± 0.088	0.11 ± 0.03	48
3-Methylisoquinoline	0.082 ± 0.025	0.035 ± 0.017	43
Xanthine	0.0072 ± 0.0028	0.012 ± 0.0057	168
G75 Fraction			
Phthalazine	0.65 ± 0.23	0.27 ± 0.17	41
Phenanthridine	0.25 ± 0.06	0.14 ± 0.09	57
3-Methylisoquinoline	0.098 ± 0.034	0.034 ± 0.02	35
Xanthine	0.0093 ± 0.0036	0.0085 ± 0.0036	91
DEAE Fraction			
Phthalazine	2.42 ± 0.83	0.5 ± 0.16	21
Phenanthridine	0.7 ± 0.29	0.2 ± 0.07	29
3-Methylisoquinoline	0.53 ± 0.27	0.14 ± 0.006	26

† The values are given as means ± S.D. for the number of animals examined (N).

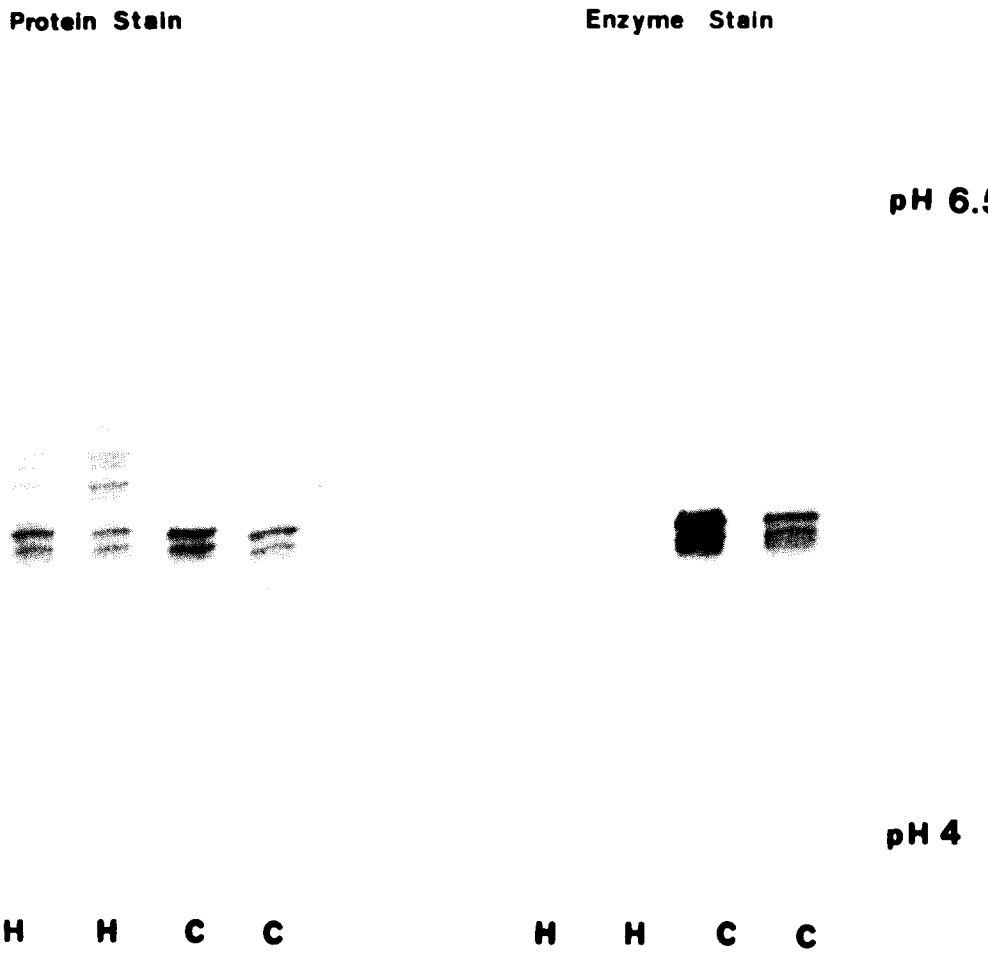

Fig. 1. Analysis of DEAE-cellulose enzyme fractions by isoelectric focusing on agarose gels. Gels were stained for protein with 0.5% Page Blue 83 and aldehyde oxidase activity was detected by incubating gels for 5 min at 37° in the dark in a solution containing 20 ml 1.0 M potassium phosphate buffer pH 7.8, 3 ml 1 mM Nitroblue tetrazolium, 3 ml 0.1 mM phenazine methosulphate and 5 ml 6 mM phthalazine: H, enzyme fractions from rabbits administered hydralazine; C, enzyme fractions from control rabbits.

Figure 1 shows the DEAE-enzyme fractions after isoelectric focusing, stained for protein and enzyme activity (see Materials and Methods). In each case, the samples stained for protein showed two or three major bands corresponding to aldehyde oxidase activity along with a number of minor contaminating proteins. In contrast, these major bands showed considerably more activity towards substrates in control enzyme fractions than in enzyme samples from hydralazine treated rabbits. Phthalazine was used as the substrate when staining for aldehyde oxidase activity although similar results were obtained using either phenanthridine or 3-methylisoquinoline. As all the enzyme solutions were initially diluted to give a protein concentration of 1 mg/ml and gave similar electrophoretic patterns when stained for protein, the variation in substrate activity cannot be attributed to differences in the amount of protein present in the samples. Consequently, these results indicate that aldehyde oxidase is still present in the liver of hydralazine treated rabbits but predominantly in an inactive form.

Interaction of hydralazine with the molybdenum hydroxylases in vitro

Hydralazine is extremely unstable in alkaline solution [21] and even in pH 7.4 buffer 30–50% decomposition may occur within 30 min [17]. Therefore, all incubations with either enzyme were performed in 0.067 M potassium phosphate buffer pH 6 as described in Materials and Methods.

There was no visible reaction between hydralazine and xanthine oxidase; neither did the addition of 0.1 mM hydralazine alter oxidation of xanthine monitored at 295 nm. Therefore hydralazine does not inhibit xanthine oxidase *in vitro* or *in vivo*. Similarly, hydralazine did not appear to be a substrate for aldehyde oxidase at pH 6. However, the rate of oxidation of other substrates was dramatically decreased in the presence of the compound. The effect of varying concentrations of hydralazine on the oxidation of 50 μM methotrexate is shown in Fig. 2. Similar results were obtained using purine as substrate, i.e. hydralazine caused a rapid progressive

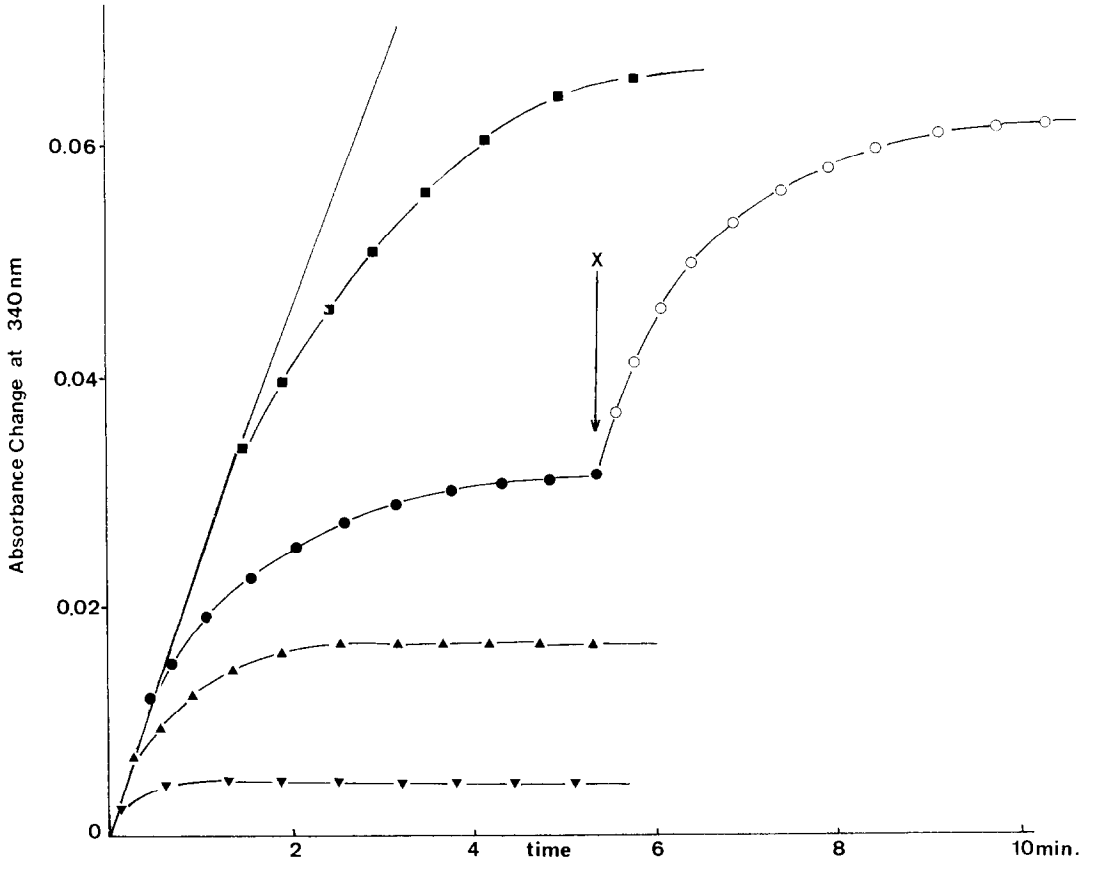

Fig. 2. Effect of hydralazine hydrochloride upon the oxidation of methotrexate by rabbit liver aldehyde oxidase. Reaction was monitored at 340 nm in 0.067 M potassium phosphate buffer pH 6 at 37°: ————, control 50 μM methotrexate; —■—■, plus 0.1 μM hydralazine; —●—●, plus 1 μM hydralazine; —▲—▲, plus 10 μM hydralazine; —▼—▼, plus 0.1 mM hydralazine; ○—○, after addition (x) of a second aliquot of aldehyde oxidase to 50 μM methotrexate and 1 μM hydralazine.

reduction of the enzymic oxidation rate. Complete inhibition was almost immediate with 0.1 mM hydralazine and even at much lower concentrations, (e.g. 0.1 μM) the rate was reduced to zero within 5 min. The initial rate of oxidation was constant even in the presence of inhibitor. Addition of a second aliquot of enzyme to the cuvette following complete inactivation resulted in recommencement of the rate which again rapidly decreased to zero. This confirms that hydralazine is exerting its inhibitory effect via the enzyme rather than by chemical interaction with the substrate. Preincubation of aldehyde oxidase with 1 μM hydralazine for 30 min, prior to the addition to substrate, gave a progress curve with methotrexate which was indistinguishable from that obtained previously. Hence, the inhibition is not due to accumulation of an inhibitory product derived from hydralazine in the medium. The lack of effect on the initial oxidation rate coupled with the potent progressive inhibition indicates that hydralazine reacts either with a reduced form of the enzyme or the enzyme–substrate complex. Progressive inhibition of other enzymes, similar to that observed with aldehyde oxidase, occurs via various mechanisms. In some cases hydralazine is thought to chelate metals which are normally necessary for enzyme activity, e.g. hydralazine inhibits proline hydroxylase by forming labile complexes with enzyme bound Fe^{2+} [22]. Such a mechanism is possible for aldehyde oxidase as the enzyme contains both molybdenum and iron. Partial restoration of proline hydroxylase activity is achieved upon addition of Fe^{2+} to inactivated enzyme [22]. However, in the present study, preincubation with Fe^{2+} or addition of Fe^{2+}, as ferrous ammonium sulphate, did not reverse the inhibition caused by hydralazine. Thus chelation of enzyme-bound Fe^{2+} probably does not occur in this case.

Interaction of hydralazine with guinea-pig and baboon liver aldehyde oxidase

Considerable species variation exists between aldehyde oxidase catalysed oxidation, particularly with phthalazine derivatives. For example, carbazeran, a phthalazine analogue developed as a potential antihypertensive agent is effective in dogs [23] but is rapidly inactivated in man by an enzyme thought to be aldehyde oxidase [24]. Similarly, hydralazine inhibits enzyme from other species in a different manner to rabbit aldehyde oxidase.

The effect of hydralazine on the oxidation of 1 mM purine by enzyme from guinea-pig (a) and baboon

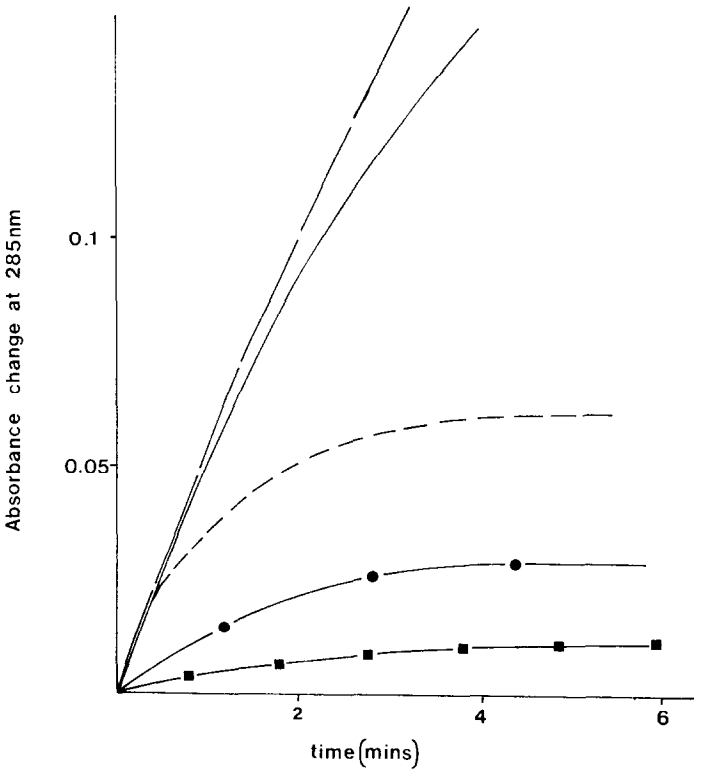

Fig. 3. Effect of hydralazine upon aldehyde oxidase from guinea pig and baboon liver. Reaction was monitored at 285 nm in 0.067 M potassium phosphate buffer pH 6 at 37°: ——, control (a) 1 mM purine with guinea pig aldehyde oxidase; —— ——, control (b), 1 mM purine with baboon liver aldehyde oxidase; – – – –, (a) plus 1 μM hydralazine; —■—■, 1 mM purine with guinea pig aldehyde oxidase after incubation of the enzyme for 15 min with 1 μM hydralazine; —●—●, (b) plus 1 μM hydralazine.

liver (b) is shown in Fig. 3. As with rabbit enzyme, no visible reaction occurred between hydralazine alone and enzyme from either source; likewise hydralazine acted as a potent inhibitor in each case. However, *preincubation* of hydralazine with guinea-pig liver enzyme considerably reduced the rate of purine oxidation indicating turnover of the enzyme had occurred during incubation and/or the formation of an inhibitory product. In addition, not only did hydralazine reduce the oxidation rate of baboon liver enzyme to zero in 2–3 min but it also decreased the initial oxidation rate to about 75% of the uninhibited rate. These results indicate that hydralazine may have an even greater inhibitory effect *in vivo* in these species and also that hydralazine itself may be oxidized by the enzyme. Further investigations along these lines are under way in our laboratory.

Interaction of hydralazine metabolites with aldehyde oxidase

The experiments performed *in vitro* show hydralazine to be a potent inhibitor of aldehyde oxidase but not xanthine oxidase which parallels the results obtained *in vivo*. However, hydralazine is extensively metabolized in rabbits [6, 7], and thus several compounds identified as metabolites were tested *in vitro* for inhibitory activity towards aldehyde oxidase: (i) hydrazine has recently been identified in rabbit urine [7]; it is known to inhibit a number of enzymes [25] including monoamine oxidase [26], the inhibition of which is similar to that exhibited by hydralazine towards aldehyde oxidase; (ii) 3-methyl-*s*-triazolo(3,4-α)phthalazine (MTP) is produced *in vivo* by the spontaneous cyclization of the *N*-acetylated metabolite of hydralazine [27]; it is one of the major metabolites of hydralazine; (iii) tetrazolo-(5,1-α)phthalazine (TP) is a minor metabolite of hydralazine which is also an inhibitor of monoamine oxidase [5].

The addition of 0.1 mM hydrazine, 0.1 mM MTP or 0.1 mM TP did not alter the oxidation rate of either purine or methotrexate. Similarly neither MTP or TP showed any substrate activity with adehyde oxidase.

Although not all hydralazine metabolites were examined in this study as possible inhibitors the only other major metabolite in rabbits appears to be a glucuronide conjugate which has not yet been fully characterized [6]. Furthermore, we have previously shown that phthalazine and 1-phthalazinone, which have also been identified after hydralazine administration in man and other experimental animals, induce aldehyde oxidase in the rabbit [1]. As both phthalazine and hydralazine appear to give phenolic products which are subsequently conjugated with glucuronic acid [20, 28] it is unlikely that this type of metabolite would inhibit aldehyde oxidase *in vivo*; therefore hydralazine itself is probably the "active"

inhibitory species *in vivo*. Oral administration of hydralazine hydrochloride (50–100 mg) to humans leads to circulating hydralazine levels of approximately 1 μM [28] thus the inhibition of aldehyde oxidase *in vivo* could have important clinical implications as the enzyme is involved in the metabolism of a number of drugs [29]. Furthermore, as the *in vitro* studies indicate the requirement for the enzyme to "turn over" before inhibition occurs, an analogous situation would also be expected to pertain *in vivo*.

Acknowledgement—C. Johnson was supported by a Research Award funded by the Pharmaceutical Society of Great Britain during this work.

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