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ELEVATION OF MOLYBDENUM HYDROXYLASE LEVELS IN RABBIT LIVER AFTER INGESTION OF PHTHALAZINE OR ITS HYDROXYLATED METABOLITE

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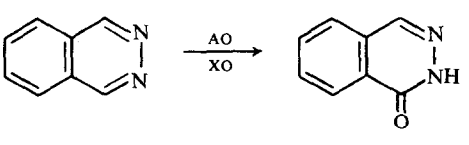
Abstract—Oral administration of phthalazine (50 mg/kg/day) or 1-hydroxyphthalazine (10 mg/kg/day) to female rabbits caused an increase in the specific activity of the hepatic molybdenum hydroxylases aldehyde oxidase and xanthine oxidase, whereas no effect on microsomal cytochrome P-450 activity was observed. The rise in the specific activity of purified aldehyde oxidase fractions was accompanied by a similar increase in molybdenum content. A significant lowering of the K_m value for phthalazine was demonstrated with enzyme from treated rabbits whereas K_m values for structurally similar substrates such as isoquinoline were unchanged from control values. Iso-electric focusing of DEAE-cellulose fractions showed the presence of an additional band of activity indicating that genuine induction of aldehyde oxidase had occurred in rabbits treated with phthalazine or 1-hydroxyphthalazine.

The occurrence of the 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) has been established for a long time [1, 2]. However, it is only recently that they have been shown to have a wide substrate specificity [3] and their potential role as drug metabolizing enzymes has been realized, e.g. xanthine oxidase rapidly oxidizes allopurinol to give a potent inhibitor [4] and aldehyde oxidase is responsible for the inactive metabolite, 7-hydroxymethotrexate, from the cytotoxic drug methotrexate [5].

As with other drug metabolizing enzymes such as cytochrome P-450, it is important to know what controlling factors govern the levels of these enzymes and whether they can be induced or inhibited *in vivo*. Many compounds stimulate their own metabolism by inducing a specific form of cytochrome P-450 [6]. Similarly, xanthine oxidase levels are elevated when mice are administered with xanthine [7]. Enzyme levels are also higher under stress conditions. Thus liver disease, administration of ethanol and exposure of animals to various infectious organisms [8–10] all lead to increased xanthine oxidase activity. Levels of aldehyde oxidase are genetically controlled but within each class the enzyme levels are regulated by the endocrine system, e.g. testosterone causes elevation of enzyme levels [11].

Aldehyde oxidase catalyses the rapid oxidation *in vitro* of 2,3-diazanaphthalene (phthalazine) to its 1-hydroxy-derivative [12].



Phthalazine is also a poor substrate for xanthine oxidase [12]. This paper therefore describes the interaction of phthalazine with aldehyde oxidase *in vivo* and also the effect on xanthine oxidase and the microsomal cytochrome P-450 system. Induction of other enzyme systems by similar heterocycles has been demonstrated. Thus quinoxaline (1,4-diazanaphthalene) derivatives cause an increase in cytochrome P-450 levels [13] and epoxide hydratase is induced when rats are treated with ethoxyquin [14].

MATERIALS AND METHODS

Reference compounds. Phthalazine and phenanthridine were purchased from Aldrich Chemical Co., xanthine was obtained from Koch-Light Laboratories Ltd. and 1-hydroxyphthalazine was synthesized according to the method of Dennis *et al.* [15].

Pretreatment of rabbits. Phthalazine (50 mg/kg/day) or 1-hydroxyphthalazine (10 mg/kg/day) was administered orally to female white New Zealand rabbits for seven days then the solutions replaced by drinking water for 24 hr. The animals were then sacrificed, and the livers were removed and placed in 1.15% potassium chloride solution at 4°.

Preparation and assay of enzyme fractions. The livers were homogenized in 1.15% potassium chloride solution and centrifuged at 10,000 *g* for 30 min at 4°. The 10,000 *g* supernatant was centrifuged at 100,000 *g* for 1 hr at 4° to isolate the microsomes or treated as described below (ii) to give partially purified aldehyde oxidase.

(i) The microsomal sediment was suspended in a mixture of 1.15% potassium chloride and 0.067 M phosphate buffer pH 7 (50:50).

Microsomal activity was estimated by measuring the extent of O-demethylation of *p*-nitroanisole (0.8 mM) occurring in 30 min utilizing the increase in absorbance at 420 nm due to *p*-nitrophenol formation [16]. Binding spectra were determined using microsomal suspensions with a protein concentration of 2 mg/ml, adding the compound (1 mg) and recording the spectrum from 340 to 480 nm.

(ii) Partially purified aldehyde oxidase was prepared from the 10,000 *g* supernatant by ammonium sulphate fractionation followed by chromatography on Sephadex G75 and DEAE-cellulose.

The 10,000 *g* supernatant was heated at 55–60° for 10 min on a water bath. The suspension was rapidly cooled and heat-precipitated protein was removed by centrifugation at 15,000 *g* for 45 min at 4°. Sufficient solid ammonium sulphate was added to give 50% saturation, the solution stirred slowly for 20 min at 4° and the precipitate collected by centrifugation at 6000 *g* for 30 min. The partially purified enzyme was dissolved in 10–15 ml of 0.1 mM EDTA and eluted with 0.1 mM EDTA from a Sephadex G75 column (60 × 2.2 cm) previously washed with several column volumes of distilled water. The fractions of eluate corresponding to aldehyde oxidase activity were pooled and concentrated by ultrafiltration. The resulting concentrate (approximately 5 ml containing 300 mg protein) was applied to a DEAE-cellulose column (60 × 1.6 cm), previously equilibrated with 5 mM potassium phosphate buffer pH 7.8, and eluted stepwise with 200 ml 0.1 M followed by 200 ml 0.2 M potassium phosphate buffer pH 7.8. Any xanthine oxidase present eluted with the 0.1 M buffer whereas aldehyde oxidase eluted with 0.2 M buffer. Active fractions were identified, combined and concentrated by ultrafiltration to give approximately 2 ml of purified aldehyde oxidase. The specific activity of enzyme fractions was determined at 37° in 67 mM potassium phosphate buffer containing 0.13 mM EDTA using the following methods:

(a) By monitoring the reduction of 1 mM potassium ferricyanide at 420 nm as described previously [12] using either 1 mM phthalazine or 0.5 mM 3-methylisoquinoline as a substrate.

(b) By directly measuring the appearance of product, utilizing oxygen as the electron acceptor. The increase in absorbance either at 322 nm due to the production of 6-phenanthridone from phenanthridine (50 μm) or at 295 nm arising from the formation of uric acid from xanthine (50 μm) was followed. Protein estimations were carried out using the Bradford method [17].

Determination of kinetic constants. Michaelis–Menten constants for substrates were determined using aldehyde oxidase purified to the $(NH_4)_2SO_4$ stage and bovine milk xanthine oxidase (24 mg/ml) from Sigma Chemical Co. The method used was that described by Stubley *et al.* [12]. The initial rates of phthalazine oxidation were also measured in the presence of a fixed concentration of inhibitor (1-hydroxyphthalazine) and the mode of inhibition and subsequently the inhibitor constants were determined by examination of double reciprocal plots derived from these data (see Fig. 1).

Measurement of the molybdenum content of enzyme fractions. The molybdenum content of DEAE-cellulose fractions of aldehyde oxidase was determined by electrothermal atomic absorption spectroscopy using a method of standard additions. A Pye Unicam SP9-01 Digital Flameless Atomiser fitted with a pyrolytically coated carbon graphite tube was used in conjunction with a Pye Unicam SP 1950 Atomic absorption spectrophotometer connected to a Deuterium Lamp Background Connector and Rapid Response Kit. Standard molybdenum solutions were prepared by dissolving 99% molybdenum wire in a mixture of concentrated nitric/concentrated hydrochloric acid (3:1) and diluting to give the required concentrations.

Analytical electrofocusing of enzyme samples. Electrofocusing was carried out using an LKB Multiphor 2117 chamber connected to an LKB 2197

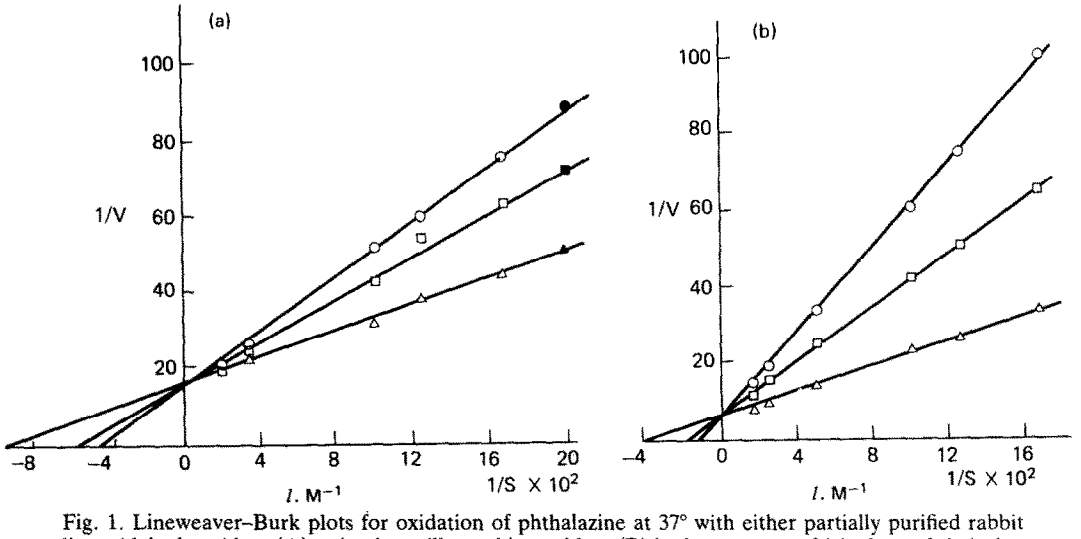


Fig. 1. Lineweaver–Burk plots for oxidation of phthalazine at 37° with either partially purified rabbit liver aldehyde oxidase (A) or bovine milk xanthine oxidase (B) in the presence of 1-hydroxyphthalazine (I) (△, ▲, [I] = 0, □, ■, [I] = 3 mM, ○, ●, [I] = 6 mM).

power supply and a circulating cooling water bath maintained at 10°. Agarose plates (0.5 mm) containing carrier ampholines, pH 4.0–6.5 were prepared according to the LKB instruction on leaflet 1818-A.

DEAE-cellulose enzyme fractions from the livers of control and treated rabbits were diluted to give protein concentrations of 1 mg per ml and 10 μl aliquots applied to small squares of filter paper (5 × 5 mm) on the surface of the agarose plate. The plate was focused at a constant power, with an initial voltage of 500 V, for 1 hr, then the power was increased by 50% and the plate focused for a further 10 min.

Protein activity in the gels was stained with 0.5% Page Blue 83 and enzyme 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 Nitrobluetetrazolium, 3 ml 0.1 mM phenazine methosulphate and 5 ml substrate solution (either 6 mM phthalazine, 3 mM 3-methylisoquinoline or 0.3 mM phenanthridine). After staining for activity the gels were dried and photographed.

RESULTS

Effect of phthalazine on microsomal activity

The effect of phthalazine administration upon the microsomal activity of rabbit liver is shown in Table 1. There is no significant difference between the liver weights of phthalazine-treated or control rabbits whereas a 20–40% increase in weight normally occurs after treatment with agents such as phenobarbitone which induce microsomal monooxygenase activity [18].

Furthermore, there is no significant increase in the percentage conversion of *p*-nitroanisole to *p*-nitrophenol by the 10,000 *g* supernatant fraction nor is there any change in the Type II binding spectra of either aniline or phthalazine with the microsomal suspension. Inducers of the microsomal system usually cause an increase in binding spectra; phenobarbitone accentuates both Type I and Type II binding spectra [19] whereas the 'relative inductive potency' of polycyclic hydrocarbons is directly related to changes in Type II binding spectra [20].

Phthalazine is not oxidized by the microsomal system *in vitro* [12, 21] and these results also suggest that very little interaction between the two occurs *in vivo*. Studies carried out using the closely related diazanaphthalene quinoxaline have shown that high concentrations of some 2,3-disubstituted quinoxalines cause an increase in rat liver microsomal activity although the parent compound is ineffective in this respect [13].

Interaction of phthalazine with molybdenum hydroxylases

The specific activities of the two molybdenum-containing enzymes, aldehyde oxidase and xanthine oxidase, were determined by comparing the maximal oxidation rate of several substrates from both phthalazine-treated and control animals. Phenanthridine is a specific substrate for aldehyde oxidase with a very low K_m value [22] whereas xanthine is oxidized exclusively by xanthine oxidase with a K_m of <1 μM [3]. Phthalazine was employed to determine whether pretreatment of the rabbits altered the rate of oxidation of this compound by aldehyde oxidase in a different manner to that of other substrates. Although phthalazine is a substrate for both enzymes the contribution from xanthine oxidase to the oxidation rate of this compound is likely to be negligible because the K_m value is 30 times higher than that with aldehyde oxidase [12] and there is very little xanthine oxidase present in rabbit liver. Treatment of the rabbits with phthalazine resulted in a 2- to 3-fold increase in aldehyde oxidase activity, measured using either phenanthridine or phthalazine, for each of the enzyme fractions tested (see Table 2). As this increase remained relatively constant throughout the purification procedure the apparent inductive effect does not appear to be due to a modifier which could be removed during purification. Similar results were also obtained using 3-methylisoquinoline as a substrate.

The level of xanthine oxidase in rabbit liver is very low compared to that of aldehyde oxidase and thus any results are less reproducible. However, the xanthine oxidase activity of the ammonium sulphate fraction was again twice that of the control although the difference in the G75 fraction was less marked.

Interaction of 1-hydroxyphthalazine with molybdenum hydroxylases

As phthalazine is a substrate for both aldehyde oxidase and xanthine oxidase *in vitro* [12] it is conceivable that 1-hydroxyphthalazine may be produced *in vivo* in rabbits by either or both enzymes. In fact small quantities of 1-hydroxyphthalazine have been detected in urine of rabbits treated with phthalazine [21], although further metabolism also occurs. Therefore 1-hydroxyphthalazine was also tested as a possible inducer of the molybdenum hydroxylases to determine if the increase in their activity during phthalazine administration was due to the compound itself or its hydroxylated metabolite. The results are

Table 1. Effect of phthalazine administration on microsomal activity of rabbit liver

Rabbit liver	Liver weight	% conversion of *p*-nitro-anisole (0.8 mM) in 30 min	*Absorbance change/mg protein	
	(g)		Aniline	Phthalazine
Control	87 (N = 7)	9.4 ± 4.1 (N = 4)	0.086 ± 0.012 (N = 3)	0.064 ± 0.013
Phthalazine treated	99 (N = 7)	10.1 ± 1.39 (N = 4)	0.097 ± 0.093 (N = 4)	0.062 ± 0.007

* Change in absorbance between λ_{max} and λ_{min} in Type II difference spectrum.

Table 2. Effect of phthalazine and 1-hydroxyphthalazine administration upon rabbit liver molybdenum hydroxylases

Fraction/ substrate	Specific activity (μmol/min/mg protein)			Specific activity after 1-hydroxyphthalazine treatment	
	Control (N = 7)	Phthalazine-treated (N = 7)	% Control	(N = 4)	% Control
Ammonium sulphate fraction					
Phenanthridine	†0.23 ± 0.09	0.64 ± 0.48	278	0.56 ± 0.29*	242
Phthalazine	0.59 ± 0.25	1.46 ± 0.80	246	1.09 ± 0.46*	183
Xanthine	0.0072 ± 0.0028	0.016 ± 0.0029	226	0.017 ± 0.0035	240
G75 fraction					
Phenanthridine	0.25 ± 0.06	0.76 ± 0.3	291	1.04 ± 0.9*	419
Phthalazine	0.65 ± 0.23	1.52 ± 0.60	232	1.86 ± 1.6*	283
Xanthine	0.0093 ± 0.0036	0.016 ± 0.011	173	0.31 ± 0.02*	330
DEAE fraction					
Phenanthridine	0.7 ± 0.2	1.78 ± 1.12	254	1.74 ± 0.8	248
Phthalazine	2.42 ± 0.83	5.34 ± 2.73	221	4.1 ± 1.1	170

†The values are given as *means* ± S.D. for the number of animals examined (N).
Each activity following treatment with phthalazine or 1-hydroxyphthalazine was significantly different from its corresponding control to at least $P < 0.05$ but only significant to $P < 0.1$ for samples * using a two-tailed Student's *t*-test.

also presented in Table 2. Pretreatment with 1-hydroxyphthalazine also caused significant increases in both molybdenum hydroxylase activities. This compound was administered at one-fifth of the phthalazine dose, yet a similar rise in enzyme activity was observed. Thus it would appear that 1-hydroxyphthalazine has a greater effect on molybdenum hydroxylase levels. Furthermore, when phthalazine was administered to rabbits at a concentration of 5 mg/kg daily for 21 days the increase in aldehyde oxidase activity was slight and not significantly different from control values. These results indicate that 1-hydroxyphthalazine, arising from the molybdenum hydroxylase-mediated oxidation of phthalazine, may be the cause of the inductive effect *in vivo*.

1-Hydroxyphthalazine is not a substrate for either aldehyde oxidase or xanthine oxidase [21], therefore, as it would be expected that some interaction with these enzymes must occur to cause induction, the compound was tested for inhibitory activity against partially purified aldehyde oxidase (ammonium sulphate fraction) and bovine milk xanthine oxidase. Figure 1 (a) and (b) show that 1-hydroxyphthalazine is a *competitive* inhibitor of phthalazine oxidation catalysed by either aldehyde oxidase or xanthine oxidase with K_i values of 4.19 mM ($c = 0\ 999$) and 4.18 mM ($c = 0.999$) respectively. Thus although the activity of phthalazine towards aldehyde oxidase differs markedly from that towards xanthine oxidase, the oxidation product appears to inhibit both enzymes to a similar extent. However, an inhibitor constant in the order of 4 mM indicates that 1-hydroxyphthalazine is not particularly effective as an inhibitor.

Determination of the molybdenum content of aldehyde oxidase fractions

Aldehyde oxidase and xanthine oxidase are complex dimeric enzymes containing 2 atoms molybdenum, 2 molecules FAD and 8 non-haem iron atoms complexed with sulphur [23]. Various inactive forms lacking molybdenum, FAD or an essential cyanolysable sulphur atom have been detected in preparations along with fully active enzyme [24–26]. In addition the molybdenum has been postulated to be present in the liver as a co-factor common to other molybdenum-containing enzymes such as sulphite oxidase [27]. In order to determine whether the rise in specific activity was accompanied by an increase in molybdenum content electrothermal atomic absorption spectroscopy was used to measure Mo-concentrations of DEAE-fractions. The low amounts of xanthine oxidase present precluded the measurement of the Mo content (μg Mo/mg protein) of this enzyme. The results are presented in Table 3.

A doubling in the Mo content of the enzyme fractions was observed following treatment with phthalazine and a less but still statistically significant rise in Mo levels occurred when 1-hydroxyphthalazine was administered. The observed increase in Mo content is of the same order as that in specific activity, which indicates that new enzyme may have been synthesized. Therefore, the rise in specific activity is probably not due to activation of a non-functional form of aldehyde oxidase which is

Table 3. Effect of administration of phthalazine and 1-hydroxyphthalazine on molybdenum content of purified aldehyde oxidase fractions

	Mo content (μg Mo/mg protein)	% Control	Activity / Mo content
Control rabbit (N = 3)	*0.38 ± 0.06	(100)	5.44
Phthalazine 50 mg/kg for 7 days (N = 5)	0.78 ± 0.34 (P < 0.05)†	203	6.98
1-hydroxyphthalazine 10 mg/kg for 7 days (N = 3)	0.46 ± 0.24 (P < 0.1)	121	6.89

* Values are quoted as means ± S.D.
† Level of significance.

Table 4. Apparent Michaelis–Menten constants for aldehyde oxidase from control and treated rabbits

Substrate	K_m values at pH 7 and 30° (M) Control	P-treated	1-OHP-treated
Phthalazine	1.1×10^{-4}	$<10^{-5}$	$\ll 10^{-5}$
Isoquinoline	2×10^{-4}	2.4×10^{-5}	—*
3-Methylisoquinoline	8.6×10^{-5}	1.6×10^{-4}	1.3×10^{-4}

* Not determined.
P = Phthalazine; 1-OHP = 1-hydroxyphthalazine.

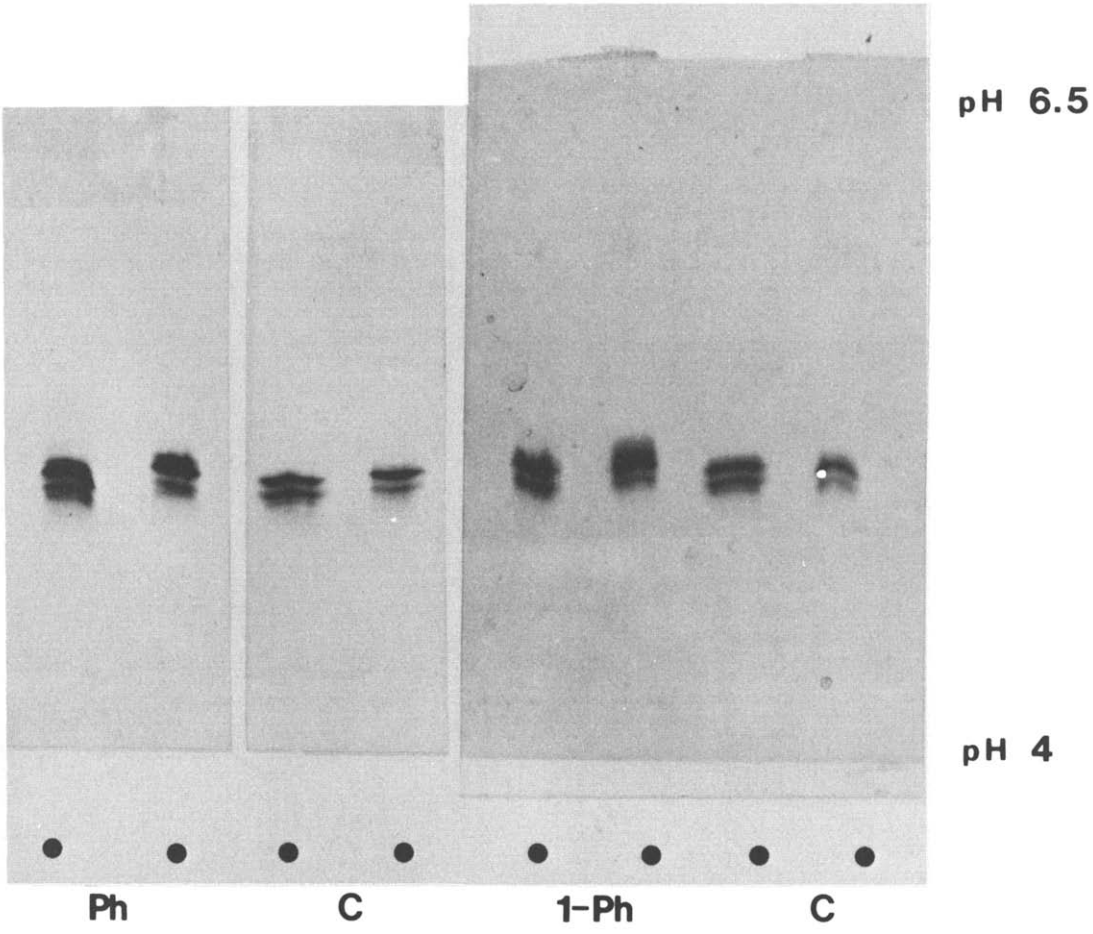


Fig. 2. Analysis of purified aldehyde oxidase (DEAE-cellulose fractions) by isoelectric focusing on agarose gels. Gels were stained for enzyme activity with 6 mM phthalazine, 0.1 mM phenazine methasulphate and 1 mM Nitroblue tetrazolium. C = enzyme fractions from control rabbits. Ph = enzyme fractions from rabbits administered phthalazine. 1-Ph = enzyme fractions from rabbits administered 1-hydroxyphthalazine.

also present in control enzyme fractions. However, this does not rule out the possibility of the incorporation of a molybdenum co-factor into existing apoenzyme which would not be expected to co-elute from the DEAE-cellulose column.

Kinetic properties of induced enzyme

As the rise in specific activity may only reflect an increase in the number of enzyme molecules, i.e. in the observed V_{max}, K_m values for phthalazine, 3-methylisoquinoline and isoquinoline were also determined using purified aldehyde oxidase fractions from control and treated rabbits. The results in Table 4 show that the Michaelis–Menten constants calculated for phthalazine with enzyme from either phthalazine or 1-hydroxyphthalazine-treated rabbits are at least 10 times lower than that obtained with control enzyme. This is in contrast to the results with isoquinoline or its 3-methylanalogue where the tendency is for the K_m values to increase with enzyme from treated rabbits. Aldehyde oxidase catalyses the oxidation of a wide range of diverse substrates [3], yet this observation indicates that a new form of the enzyme with enhanced specificity for only one substrate, i.e. phthalazine, has been induced when rabbits are administered with this compound or its hydroxylated metabolite.

Comparison of enzyme samples using iso-electric focusing

A qualitative examination of the enzyme fractions was carried out using iso-electric focusing according to the procedure described in the "Methods" section.

The electrophoretic patterns from DEAE-cellulose fractions from control, phthalazine and 1-hydroxyphthalazine-treated rabbits are shown in Fig. 2. The plate was stained for aldehyde oxidase activity using a modification of the technique used by Holmes [28]. The same profile was observed in each case when either phenanthridine, phthalazine or 3-methylisoquinoline was used as a substrate. Plates stained for protein activity showed the major protein bands to be present in positions corresponding to enzyme activity with a number of other bands due to contaminating proteins also apparent. There were consistently 2–3 bands of aldehyde oxidase activity in all samples from control rabbits, the IpH of these bands was found to be approximately 5. The apparent presence of a number of isoenzymes in normal rabbit liver is currently under investigation in this laboratory, the details of which will be published separately.

In addition to the bands present in the control samples a further closely related band of activity was noted in all the samples from both sets of treated rabbits, irrespective of which substrate was employed in the staining process. The appearance of another band, with a slightly higher isoelectric point, can be attributed to a new form of the enzyme arising during phthalazine and 1-hydroxyphthalazine treatment. This observation correlates well with the results of the K_m determinations which also indicate that a novel form of the enzyme may have been induced.

DISCUSSION

The results of this study indicate that, in common with other drug metabolizing enzymes, aldehyde oxidase may be an inducible enzyme. There are no previous reports relating to aldehyde oxidase induction. However, the concomitant rise in molybdenum concentration and activity towards substrates suggest that genuine enzyme induction is occurring in this case. The inductive effect appears to be common to both rabbit molybdenum hydroxylases. Although the low levels of xanthine oxidase in rabbit liver precluded the measurement of molybdenum levels corresponding to this enzyme, a rise in specific activity, similar to that obtained with aldehyde oxidase, was observed. In contrast there was no evidence to suggest that phthalazine treatment had any effect on the microsomal cytochrome P-450 system.

Furthermore, the "induced" form of aldehyde oxidase differs from control enzyme in at least two respects. An additional active variant was detected by isoelectric focusing in all "induced" samples under various substrate conditions. Secondly, enzyme samples from treated rabbits had a higher affinity towards phthalazine than control enzyme but still retained the same efficiency towards other substrates. Thus the increased activity towards phthalazine arises from combined effects on both K_m and V_{max} values whereas the rise in specific activity observed with other substrates results from elevated V_{max} values due to higher enzyme levels. This phenomenon is not unusual for drug metabolizing enzymes with multiple substrate specificity such as microsomal cytochrome P-450. There are many reports of compounds stimulating their own metabolism in particular but still inducing cytochrome P-450 which is active towards other substrates [6].

Finally it is not thought that the inductive effect is due to phthalazine itself as it is extensively metabolized in rabbits [21] but may be caused by one of its metabolites, possibly 1-hydroxyphthalazine, which we have shown to be a competitive inhibitor of both molybdenum hydroxylases.

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