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DICHLORO-*p*-NITROANISOLE *O*-DEMETHYLASE—II. EVIDENCE FOR SEPARATE ETHANOL INHIBITED AND PHENOBARBITAL-INDUCIBLE ENZYMES

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Abstract—Some properties of the monooxygenase activity measured in the recently described dichloro-*p*-nitroanisole *O*-demethylase assay were investigated in rat liver microsomes. Enzyme activity in control microsomes was inhibited by ethanol (I_{50} = 5 mM). The small slope of the inhibition curve was interpreted as evidence for a heterogenous enzyme system, with at least one enzyme being highly sensitive to ethanol inhibition. A number of other water-miscible organic solvents also inhibited the reaction, tetrahydrofuran being most efficient (I_{50} = 0.6 mM). Tween 80 gave only weak inhibition at concentrations up to 4 mg/ml. Vinyl chloride was a strong inhibitor. Phenobarbital pretreatment of the rats increased *O*-demethylase activity at least 24-fold, and made the enzyme highly sensitive to metyrapone inhibition but not to ethanol. P-448 was not involved in the reaction, since 3-methylcholanthrene pretreatment, or addition of 7, 8-benzoflavone, produced no effect. Only small differences were found between the enzyme from male and female rats.

Several assays for drug metabolizing enzymes in the liver have been established. In common use is the *p*-nitroanisole *O*-demethylase assay [1], in which a coloured product is determined spectrophotometrically. This laboratory recently published a new assay, based on the chlorinated analogue to *p*-nitroanisole, 1,3-dichloro,2-methoxy,5-nitrobenzene (dichloro-*p*-nitroanisole) [2]. This assay demonstrates several advantages: the product, 1,3-dichloro,2-hydroxy,5-nitrobenzene (dichloro-*p*-nitrophenol), is fully dissociated at physiological pH values, and its colour is thus insensitive to moderate changes of pH in the medium. More important, the product is not further metabolized by conjugating enzymes, making the assay directly applicable in systems where such enzymes are active, e.g. in isolated hepatocytes.

The demethylation of dichloro-*p*-nitroanisole is mediated by a microsomal monooxygenase, probably cytochrome P-450 [2]. This class of oxygenases is heterogenous, with several cytochrome P-450 species having different substrate specificities and responses to specific inducers and inhibitors [3–5]. This work further characterizes the dichloro-*p*-nitroanisole *O*-demethylase, using some well-known inducers and inhibitors of drug metabolism.

The effects of a number of water-miscible organic solvents on dichloro-*p*-nitroanisole *O*-demethylase were also investigated, since initial attempts to add the substrate as an ethanol solution revealed that ethanol inhibits the reaction.

MATERIALS AND METHODS

Chemicals. Dichloro-*p*-nitroanisole and dichloro-*p*-nitrophenol were synthesized as described [2]. Tetrahydrofuran was used as supplied, or immediately after removal of contaminating peroxides on an aluminium oxide column. The absence of peroxides in the eluate was demonstrated by the inability to oxidize iodide to iodine. No difference in the effects on metabolism was seen between the purified and the crude compound. Vinyl chloride gas (KemaNord AB) was handled with a syringe and diluted with air in glass flasks with rubber membranes. The precision of the final concentration was checked by adding a small amount of [^{14}C] vinyl chloride (NEN) at the start of the procedure. Biochemicals used in the assays were as published [2]. All other chemicals were of the highest purity available, and used without further purification.

Microsomes were prepared from livers of approximately 250 g strain R rats (a Wistar strain). Rats were fed on Forss R3 pellets and water *ad lib.*, but were starved overnight before they were killed. Phenobarbital, 80 mg/kg, was injected i.p. for three consecutive days, the last injection being given the day before the experiment. 3-Methylcholanthrene, 20 mg/kg, was given for two days by the same route; on the third day only vehicle was injected. Controls received vehicle only (saline or peanut oil, respectively; no difference was seen between the two kinds of control microsomes). The microsomal fraction was centrifuged through a sucrose layer as previously described [2, 6].

Enzyme assays. Microsomes were incubated for 30 min at 37° in 0.07 M potassium phosphate buffer containing 1% bovine serum albumin (w/v), and an NADPH-generating system [2]. The 2.5 ml assay volume contained approximately 1 mg microsomal protein. Under these conditions the assay was linear with time and amount of microsomal protein added [2]. Before incubation, dichloro-*p*-nitroanisole (1 μmole) in ethanol was added to each tube, and the solvent evaporated. Where indicated 7,8-benzoflavone was added in the same fashion. To obtain low background values the

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dichloro-*p*-nitrophenol formed was assayed after extraction, as previously described [2]. Standards and blanks (without the NADPH-generating system) were treated as above. Protein was determined as described by Lowry *et al.* [7].

Calculation of I_{50}*. Assuming one enzyme, simple Michaelis–Menten kinetics and reversible inhibition it can be shown that

$$\log [(1-V_r)/V_r] = \log I - \log I_{50}. \quad (1)$$

This equation holds whether inhibition is competitive or noncompetitive; if competitive, $I_{50} = K_i(1 + S/K_M)$, and if noncompetitive $I_{50} = K_i$. From equation (1) it follows that when $\log [(1-V_r)/V_r]$ is plotted against log I, a straight line with the slope + 1 is obtained, intercepting the abscissa at $\log I = \log I_{50}$. A similar, but nonlogarithmic, linear relation for determining I_{50} has recently been derived [8]. In practice, we often found the slope to be smaller than the theoretical value (this will be discussed further). Introducing the empirical constant α we obtain

$$\log [(1-V_r)/V_r] = \alpha(\log I - \log I_{50}), \quad (2)$$

where ideally $\alpha = 1$. I_{50} and α were determined by linear regression using the least-squares method. Means and standard deviations were calculated from several independently determined values as indicated in the tables.

RESULTS

Differences between animals of different pretreatment and sex in the activity of dichloro-*p*-nitroanisole *O*-demethylase, and in its sensitivity to some inhibitors are summarized in Table 1. The enzyme in liver microsomes from control rats is characterized by its unusually high sensitivitiy to ethanol inhibition ($I_{50} = 5.3$ mM). It is also inhibited by metyrapone ($I_{50} = 2.1$ mM). 7,8-Benzoflavone has no effect, even at the highest concentration tested (40 μM).

Phenobarbital pretreatment dramatically increased the activity and changed the qualitative properties of the enzyme (Table 1.) Although the induced microsomes were diluted 6–7 times, a substantial part of the substrate was consumed during the incubation. Thus the 24-fold increase in specific activity shown in Table 1 might be underestimated. After phenobarbital pretreatment the dichloro-*p*-nitroanisole *O*-demethylase was only inhibited at very high ethanol concentrations ($I_{50} = 0.41$ M), while the sensitivity to metyrapone was increased almost 400-fold ($I_{50} = 5.4$ μM).

In contrast to phenobarbital pretreatment, 3-methylcholanthrene pretreatment produced minor effects (Table 1).

The difference in properties of the *O*-demethylase enzyme obtained from male and female rats was small, the enzyme obtained from females being slightly more sensitive to inhibition with ethanol (Table 1, $I_{50} = 2.1$ mM).

Inhibition curves. Our inhibition data generally gave curved Lineweaver–Burk plots, and it was thus difficult to obtain reproducible K_M and K_i values. For this reason we have presented all inhibition data in terms of mean I_{50} values, as described in Materials and Methods.

The metabolism of dichloro-*p*-nitroanisole in control microsomes decreases very slowly with increasing concentration of ethanol, compared to what Michaelis–Menten kinetics would predict (Fig. 1a). This is similarly reflected in the low α-value (0.46) compared to the predicted value 1.00 (Table 1, *cf.* Materials and Methods). This could be explained if more than one enzyme was simultaneously metabolizing the substrate, each enzyme having a different susceptibility to inhibition. This principle is illustrated in Fig. 1a, where our data

* Abbreviations: I, inhibitor concentration; I_{50}, inhibitor concentration giving 50 per cent inhibition; V_r, relative reaction velocity = velocity with inhibitor/velocity without inhibitor; K_i, inhibitor constant; K_M, Michaelis–Menten constant; S, substrate concentration.

Table 1. Sex dependency and effects of pretreatment on dichloro-*p*-nitroanisole *O*-demethylase activity and its sensitivity to inhibitors

	Control		Phenobarbital	3-Methyl-cholanthrene
	Male	Female	Male	Male
Enzyme activity, nmoles/min. mg prot.	0.74 ± 0.27(17)	0.74 ± 0.04(3)	17.3 ± 0.0(2)	0.81 ± 0.05(5)
Ethanol inhibition				
log I_{50}	−2.28 ± 0.31(13)	−2.67 ± 0.26(3)	−0.39 ± 0.10(2)	−2.12 ± 0.22(5)
I_{50}, mM	5.3	2.1	409	7.7
α	0.46 ± 0.09(13)	0.48 ± 0.04(3)	0.73 ± 0.20(2)	0.47 ± 0.07(5)
Metyrapone inhibition,				
log I_{50}	−2.69 ± 0.66(8)	−2.83(1)	−5.27 ± 0.41(2)	−3.63 ± 0.66(5)
I_{50}, mM	2.1	1.5	0.005	0.24
α	0.52 ± 0.21(8)	0.49(1)	0.88 ± 0.03(2)	0.42 ± 0.06(5)
7, 8-Benzoflavone, % Inhibition at 40 μM	0 ± 8(8)	0 ± 7(3)	2 ± 3(2)	11 ± 7(4)

Microsomes were assayed for dichloro-*p*-nitroanisole *O*-demethylase as described in Materials and Methods. Duplicate determinations with at least 3 different concentrations of inhibitor were done in most experiments, and I_{50} interpolated as described. Inhibition with 7, 8-benzoflavone is shown at the highest concentration tested. Mean values ± SD are shown; numbers of separate experiments are indicated in parentheses.

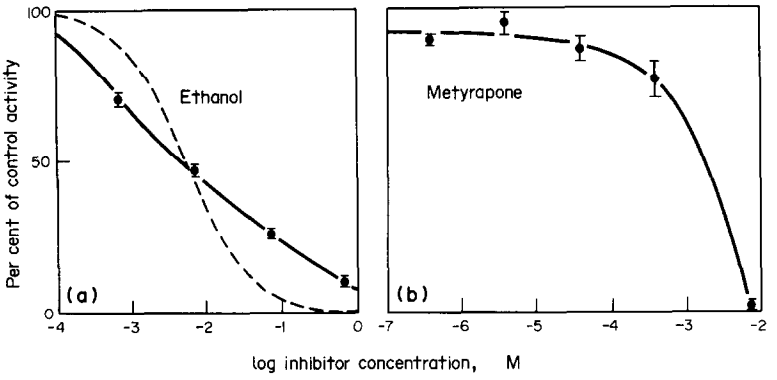

Fig. 1. Inhibition of dichloro-*p*-nitroanisole *O*-demethylase in liver microsomes from untreated male rats. Incubations were as described in Materials and Methods. (a) Inhibition with ethanol. The curves drawn are theoretical ones, the solid line assuming three enzymes with I_{50}–values 0.5, 16 and 400 mM, and contributing to 48, 28 and 24 per cent respectively of the metabolism in the absence of ethanol(see text). The broken line was calculated assuming one enzyme only, with I_{50} = 5.3 mM (Table 1). Experimental points are mean values ± SDM from 13 experiments with duplicate determinations. (b) Inhibition with metyrapone. Mean values ± SDM from 8 experiments with duplicate determinations.

are compared with the theoretical curve for one enzyme with I_{50} = 5.3 mM (broken line), and with a theoretical curve, assuming three different enzymes with I_{50} = 0.5 mM, 16 mM and 400 mM, and contributing to 48, 28 and 24 per cent respectively of the metabolism in the absence of ethanol (solid line). The numbers used to construct the latter curve were chosen by trial and error, since the data are not detailed enough to permit a rigorous regression analysis. We were not able to obtain a good fit assuming less than three different enzymes. The proposed heterogeneity would also explain the curved Lineweaver–Burk plots.

The inhibition with metyrapone was unusual in that metabolism first decreased slowly with increasing concentration of inhibitor, then dropped abruptly to zero at 10 mM metyrapone (Fig. 1b). This concentration always gave total inhibition, irrespective of pretreatment or sex (data not shown). The variation between experiments of the effect of this inhibitor is larger than with other inhibitors tested (Fig. 1, Tables 1 and 2).

Inhibition with other substances. The effect of a number of water-miscible organic solvents on dichloro-*p*-nitroanisole *O*-demethylase in microsomes from untreated male rats is summarized in Table 2. Most of the tested substances gave inhibition curves similar to the one described for ethanol ($\alpha \approx 0.5$, Fig. 1a). they were effective at moderate concentrations (I_{50} < 50 mM), methanol being the only exception. Even the I_{50} of methanol, however, corresponded to a concentration as low as 0.8% (v/v). Tetrahydrofuran was the most potent inhibitor of the solvents.

We also investigated the effect of vinyl chloride, a carcinogen known to be metabolized by an ethanol-sensitive pathway [9], on dichloro-*p*-nitroanisole *O*-demethylase (Table 2). Vinyl chloride was found to be the most efficient inhibitor of all compounds tested. The concentration of vinyl chloride gas in the incubation mixture was calculated from air concentrations and the separately measured solubility in the albumin-containing buffer. The presence of microsomes might have further increased the solubility to some extent. However, even in the unlikely case that all the vinyl chloride was dissolved in the medium, the calculated I_{50} is still very low (0.16 mM).

Tween 80, a detergent often used as a carrier of lipophilic compounds, was also tested. Only weak inhibition of dichloro-*p*-nitroanisole *O*-demethylase was seen in the concentration range tested (0.004–4 mg/ml). At the highest concentration the enzyme activity was reduced about 20 per cent (data not shown). The

Table 2. Inhibition of dichloro-*p*-nitroanisole by water-miscible organic solvents, and by vinyl chloride

Inhibitor	Number of experiments	log I_{50} (± SD)	I_{50} (mM)	α ± SD
Methanol	2	−0.70 ± 0.02	199	0.90 ± 0.45
Acetone	2	−1.42 ± 0.37	38	0.50 ± 0.10
Dimethyl sulfoxide	2	−1.59 ± 0.22	26	0.44 ± 0.04
Sulfolan	1	−1.61	24	0.84
Ethanol	13	−2.28 ± 0.31	5.3	0.46 ± 0.09
Tetrahydrofuran	5	−3.20 ± 0.26	0.63	0.48 ± 0.10
Vinyl chloride	4	−4.75 ± 0.31	0.018	0.49 ± 0.12

Microsomes were assayed for dichloro-*p*-nitroanisole *O*-demethylase as described in Materials and Methods. Duplicate determinations with at least 3 different concentrations of inhibitors were done, and I_{50} interpolated as described. Data for ethanol are included from Table 1 for comparison.

extraction of the reaction product was affected by the detergent, and it was therefore necessary to include Tween 80 in blanks and standards.

DISCUSSION

The results presented indicate that at least two different enzymes contribute to the metabolism of dichloro-*p*-nitroanisole. In the untreated rat, much of the metabolism is mediated by an ethanol-inhibited enzyme, while in phenobarbital pretreated rats the substrate is metabolized mainly by an enzyme which shows increased sensitivity to inhibition with metyrapone, but is not inhibited by ethanol.

The existence of an ethanol-sensitive cytochrome P-450 has been suggested by others [10, 11]. The high sensitivity to tetrahydrofuran inhibition is also in agreement with the published properties of such a P-450 species [9, 11]. We estimate that this enzyme contributes about half of the dichloro-*p*-nitroanisole *O*-demethylase activity in the untreated male rat (Fig. 1a). Vinyl chloride is also metabolized by a microsomal monooxygenase, similarly inhibited by ethanol and tetrahydrofuran [9]. Thus, the inhibition of dichloro-*p*-nitroanisole *O*-demethylase by vinyl chloride could be interpreted as competitive interaction with an enzyme metabolizing both substrates. Other explanations are also possible, since vinyl chloride has been reported to cause non-specific destruction of cytochrome P-450 [12, 13]. In these studies, however, much higher concentrations of vinyl chloride were employed than we have used. The special properties of dichloro-*p*-nitroanisole *O*-demethylase are in contrast to those of *p*-nitroanisole *O*-demethylase [1], which we found to be less sensitive to inhibition with ethanol and tetrahydrofuran (I_{50} values 10 times higher, unpublished). Since all tested water-miscible organic solvents interfere with dichloro-*p*-nitroanisole *O*-demethylase, such solvents should be avoided in the assay. When it is not possible to evaporate solvents before incubation, Tween 80 might be a useful carrier for lipophilic substances, since this substance interferes little with the assay.

The absence of effects of 3-methylcholanthrene pretreatment shows that the cytochrome P-450 species (P-448) specifically induced by this polycyclic hydrocarbon [4, 14] does not contribute significantly to metabolism of dichloro-*p*-nitroanisole. This conclusion is further strengthened by the small effects of 7,8-benzoflavone, a specific inhibitor of 3-methylcholanthrene-induced metabolism [15].

Phenobarbital is often considered to be an unspecific inducer of drug metabolism, causing a quantitative increase in hepatic enzymes, without changing their qualitative properties [4, 5]. However, specific induction by phenobarbital of one cytochrome P-450 species, as shown by SDS-polyacrylamide electrophoresis and by immunological methods, has been reported [16]. An increased sensitivity to metyrapone inhibition has also been shown [11]. The unusually large increase in activity, as well as the drastically changed sensitivity to inhibitors of dichloro-*p*-nitroanisole *O*-demethylase after phenobarbital pretreatment described by us also suggest specific induction of one enzyme subgroup. The demonstrated sensitivity and specificity of the dichloro-*p*-nitroanisole *O*-demethylase assay for phenobarbital-induced changes in hepatic mixed-function oxidases could make the assay a valuable tool in studies of phenobarbital induction. The effects of phenobarbital, ethanol and metyrapone on microsomal metabolism have also been reproduced in suspensions of intact hepatocytes (unpublished results, *cf.* [9]).

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