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Characterization of the *In Vivo* Inhibition of Rat Hepatic Microsomal Aldehyde Dehydrogenase Activity by Metyrapone

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ABSTRACT. Microsomal aldehyde dehydrogenase (mALDH; EC 1.2.1.3) has been proposed to catalyze the oxidation of various aldehydic products of lipid peroxidation, but the regulation of the enzyme has not been characterized. Metyrapone administration (100 mg/kg, i.p.) produced a rapid decline in the rates of mALDH-catalyzed decanal dehydrogenation; other xenobiotics were generally without effect. Thus, a 22% decrease in activity was detected 2 hr following metyrapone administration, and 52% of the activity remained at 6 hr. The decrease in microsomal decanal dehydrogenation was also dose-dependent with 70, 43, and 12% of the control activity remaining following pretreatment with 25, 100, and 250 mg/kg metyrapone, respectively. This decrease in microsomal decanal dehydrogenase activity occurred without a change in mALDH immunoreactive protein, and metyrapone did not inhibit the activity *in vitro*. The kinetic analysis revealed similar decreases in the maximal reaction velocities (V_{max}) for both decanal and NAD in the metyrapone-treated group (200 ± 10 and 190 ± 20 nmol NADH produced/min/mg protein, respectively) compared with the untreated group (330 ± 10 and 350 ± 20 nmol NADH produced/min/mg protein, respectively), but the Michaelis constants (K_m) were unchanged. These data are consistent with the *in vivo* inactivation of a portion of the mALDH enzyme. A possible consequence of the *in vivo* inhibition of this enzyme by metyrapone could be the accumulation of toxic aldehydes in the vicinity of the microsomal membrane following lipid peroxidation. BIOCHEM PHARMACOL 51;9:1187–1193, 1996.

KEY WORDS. metyrapone; aldehyde dehydrogenase; enzyme inactivation; hepatic microsomal enzymes

Previous studies have identified numerous forms of ALDH† (EC 1.2.1.3) located in the various subcellular fractions of mammalian liver [1, 2]. Several have been purified to homogeneity, and recent documentation of the cDNA sequences has established the relatedness of the proteins and enabled a nomenclature to be derived [3, 4]. The mitochondrial, microsomal, and some cytosolic forms of ALDH are constitutively expressed in normal liver, and xenobiotic pretreatment with phenobarbital or tetrachlorodibenzo-*p*-dioxin up-regulates additional cytosolic forms not normally present in rat liver [5]. To date, the hepatic mitochondrial and cytosolic forms of ALDH have been widely studied because of their involvement in the biotransformation of acetaldehyde generated *in vivo* after ethanol ingestion [6].

Rat hepatic microsomes possess a high level of ALDH activity, but the function of this enzyme has not been explored in detail. mALDH displays a broad substrate specificity, and both aliphatic and aromatic aldehydes have been found to be substrates [7, 8]. It has been proposed that this enzyme is involved in the elimination of aldehydic products of lipid peroxidation [7, 9]. The basis for this suggestion is that optimal activity has been detected *in vitro* with medium to long chain aliphatic aldehydes [7] and that similar compounds have been detected following carbon tetrachloride– or ADP–iron-induced lipid peroxidation of microsomes [10]. The pretreatment of rats with the peroxisomal proliferator drug clofibrate has also been shown to increase mALDH activity when the substrate was benzaldehyde, phenylacetaldehyde, and nonanal [11].

The present study was undertaken to assess the inducibility of the mALDH following administration of xenobiotics to male and female rats. The principal finding to emerge from this study was that, although most typical inducers of biotransformation enzymes had no effect on dehydrogenase activity, metyrapone produced a marked decrease in mALDH-catalyzed decanal dehydrogenase activity. This occurred without any effect on mALDH protein and appears to be due most likely to post-translational modification. It is possible that this is a consequence of the interaction of metyrapone or one of its metabolites with the mALDH that results in the inactivation of a portion of the enzyme.

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† *Abbreviations:* ALDH, aldehyde dehydrogenase; mALDH, microsomal aldehyde dehydrogenase; metyrapone, 2-methyl-1,2-bis(3-pyridyl)-1-propanone; IgG, immunoglobulin G; and CYP, cytochrome P450.

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MATERIALS AND METHODS

Chemicals

Dexamethasone, triacetyloleandomycin, β-naphthoflavone, metyrapone, decanal, NAD, dimethyl sulfoxide, Tween 20, Triton X-100 and anti-rabbit IgG-peroxidase conjugate were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3-Benzoylpyridine, 3-picoline *N*-oxide, α-pyridoin, and 2,3-di-3-pyridyl-2,3-butanediol were purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Phenobarbital sodium was obtained from the Pharmacy Department at Westmead Hospital. Reagents used to visualize immunoreactive mALDH protein by enhanced chemiluminescence were obtained from Amersham (Buckinghamshire, England). Reagents for electrophoresis and immunoblotting were from Bio-Rad (Richmond, CA, U.S.A.).

Animals and Preparation of Hepatic Microsomal Fractions

Livers from male and female Wistar rats (*ca.* 10 weeks of age) were used in these experiments. In initial studies, xenobiotics were administered by intraperitoneal injection to adult male and female rats as previously described [12], except that in the case of metyrapone, a single dose (100 mg/kg) was administered and rats were killed 24 hr later. Since the inhibitory effect produced by metyrapone was similar in rats of either gender, females were used in subsequent experiments. A further group of rats was treated with a range of doses (1–250 mg/kg) while another group was killed at several times (0, 2, 4, 6 and 8 hr) after a single dose (100 mg/kg). Other compounds possessing structural features in common with metyrapone, such as 3-benzoylpyridine, 2,3-di-3-pyridyl-2,3-butanediol, 3-picoline *N*-oxide, and α-pyridoin, were administered to female rats (100 mg/kg) that were killed 24 hr later. Rats were fasted overnight and, following killing, the livers were removed, perfused with ice-cold saline, frozen in liquid nitrogen, and stored at −70°. Microsomes were prepared by differential ultracentrifugation [13] with an additional wash step to minimize contamination of microsomes with cytosol.

Assay of Decanal Dehydrogenation in Rat Hepatic Microsomes

mALDH-catalyzed decanal dehydrogenation was assayed spectrophotometrically at 340 nm, which reflects the appearance of NADH from NAD. Incubations were routinely performed in 0.1 M potassium phosphate buffer, pH 7.4, that contained EDTA (1 mM), Triton X-100 (0.009%) and NAD (1 mM). The presence of Triton X-100 did not alter the rate of dehydrogenation and has been used routinely to increase the solubility of lipophilic aldehyde substrates [9]. Substrate (decanal, 25 μM except in the determination of kinetic parameters) was introduced into the reaction in dimethylformamide (10 μL); this volume of solvent did not influence the reaction rate. The reaction was performed at 37° and was initiated by addition of microsomal protein (20–60 μg). Activity was not detected in the absence of cofactor, substrate, or protein.

Purification of mALDH

The purification of the mALDH from sodium cholate-solubilized microsomes was similar to a previously described procedure [2]. Briefly, sequential chromatography was conducted on DEAE-cellulose, 5′-AMP Sepharose 4B, and hydroxylapatite and yielded a single protein of apparent subunit molecular mass of 54 kDa (as determined by SDS–PAGE). The specific activity of this preparation (with respect to decanal as the aldehyde substrate) was enhanced 25-fold over that in microsomes, and 17% of the activity present in cholate-solubilized microsomes was recovered.

Preparation of the Anti-Rat Anti-mALDH IgG

The anti-mALDH IgG proved unsuitable for immunoblotting and was purified further on an mALDH-coupled CNBr-activated Sepharose 4B column. Briefly, the CNBr-activated Sepharose (0.3 g) was added to mALDH (3–5 mg) in Buffer A (0.1 M $NaHCO_3$ buffer, pH 8.3, containing 0.5 M NaCl). Binding of protein was achieved by shaking overnight at 4°, and residual activated groups were blocked with 0.2 M glycine buffer, pH 8.0. Excess protein was removed by successive washes with 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl and then with Buffer A. After equilibration with buffer A, the anti-mALDH IgG was applied and recycled through the affinity column overnight at 4°. The purified IgG fraction that bound to this column was eluted with potassium thiocyanate (2 mM). This immunopurified IgG was specific for the mALDH and was suitable for immunoblotting.

Immunoquantitation of mALDH in Rat Hepatic Microsomes

Rat hepatic microsomes (6 μg) were separated by electrophoresis on 7.5% polyacrylamide gels in the presence of SDS essentially by the method of Laemmli [14], except that the concentrations of glycine and Tris were doubled in all buffers [15]. After electrophoresis, proteins were transferred to nitrocellulose sheets by the method of Towbin *et al.* [16]. The nitrocellulose sheets were washed in TBS-Tween (containing 50 mM Tris, 200 mM sodium chloride, and 0.05% Tween 20, pH 7.4) for 30 min followed by TBS-Tween containing 5% powdered milk for 60 min. The sheets were incubated with the immunopurified anti-rat anti-mALDH IgG (at a concentration of 1.25 μg/mL) for 90 min. A series of wash steps was employed: first using TBS-Tween for 5 min each and then TBS-Tween containing 5% powdered milk for 10 min, followed by incubation with an anti-rabbit IgG-peroxidase conjugate (1 in 3000 dilution) in TBS-Tween containing 5% powdered milk for 60 min. Following another series of wash steps for 5 min each in TBS-Tween,

the immunoreactive protein was visualized by enhanced chemiluminescence. The nitrocellulose sheets were exposed to X-ray film (Hyperfilm MP, Amersham), and the autoradiographs obtained were analyzed by densitometry (ImageQuant, Molecular Dynamics). Protein concentrations were quantitated with a standard curve constructed with the purified protein (0–240 ng).

Statistics

Data are expressed as means ± SD. All measurements were conducted on samples from individual rats. Comparisons between two groups were made using Student's *t*-test, while comparisons between multiple treatment groups were analysed by single factor analysis of variance and Student–Newman–Keuls *q*-test.

RESULTS

Effect of Xenobiotic Pretreatment on Microsomal Decanal Dehydrogenation

As part of the present investigation of the regulation of mALDH, several xenobiotics that are also established inducers of CYP enzymes were administered to rats. Animals received phenobarbital (inducer of CYP 2B and 3A), β-naphthoflavone (CYP 1A inducer), dexamethasone (CYP 3A inducer), triacetyloleandomycin (CYP 3A inducer and inhibitor), dimethyl sulfoxide (CYP 2E1 inducer and inhibitor) or metyrapone (CYP 2B1 inducer) [17–19]. With the exception of metyrapone, none of these treatments altered the rate of microsomal decanal dehydrogenation (data not shown). Figure 1 demonstrates the pronounced 46–60% decrease in mALDH-catalyzed decanal dehydrogenation in both male and female rats that was elicited by metyrapone (at 100 mg/kg).

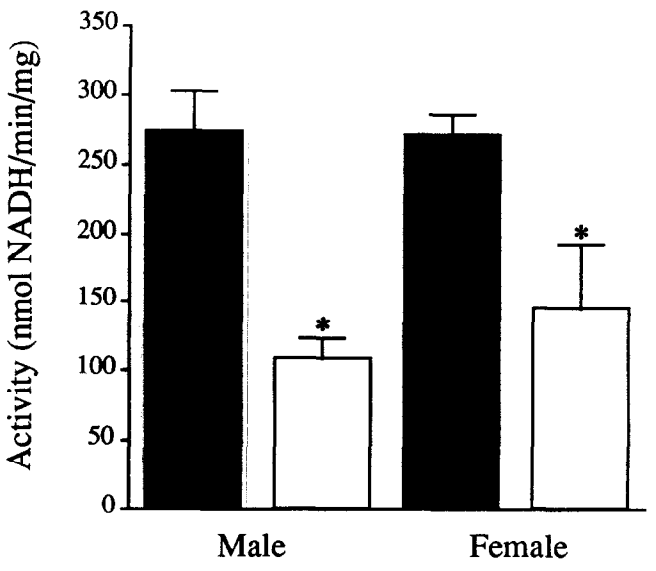


FIG. 1. Decanal dehydrogenation in untreated male and female rat hepatic microsomes (solid bars) and following the *in vivo* administration of a single 100 mg/kg dose of metyrapone (open bars). Data are expressed as means ± SD for N = 4–5 rats per group. Significant difference from the untreated group: (*) $P < 0.001$.

In view of this effect of metyrapone on microsomal decanal dehydrogenation, the microsomal content of the mALDH was determined by immunoblotting with a monospecific IgG preparation. Surprisingly, the appearance of immunoblots corresponding to the mALDH was very similar in microsomes from untreated and metyrapone-treated female rats (Fig. 2). This was confirmed by laser densitometry so that quantitation of mALDH protein content in untreated and metyrapone-treated rat liver was determined to be 23 ± 2 ng/μg microsomal protein (Table 1).

The time- and dose-dependencies of the inhibitory effect by metyrapone on mALDH activity were investigated. From Fig. 3, it can be seen that a significant decline in decanal dehydrogenation rates, to about 78% of control, occurred as early as 2 hr after a single 100 mg/kg dose of metyrapone ($N = 4$, $P < 0.05$). By 6 hr the rate of mALDH-catalyzed decanal dehydrogenation was decreased by approximately 50% from control levels and, although there was further decline at 8 hr, this appeared optimal at this concentration of metyrapone. No further decrease in decanal dehydrogenation was observed when rats were killed 24 hr after a similar dose of metyrapone.

Treatment of female rats with doses of metyrapone up to 10 mg/kg (24 hr before death) did not affect microsomal decanal dehydrogenation, but the higher doses of 25, 100, and 250 mg/kg significantly decreased decanal dehydrogenation by 30, 57, and 88% of the activity displayed in untreated rat liver ($N = 4$, $P < 0.005$ for each group; Fig. 4).

Kinetics of the Effects of Metyrapone on Decanal Dehydrogenation Activity in Rat Liver

Kinetic parameters were determined for decanal dehydrogenation in microsomes from untreated and metyrapone-treated female rats. The Michaelis constant of decanal dehydrogenation was essentially identical in hepatic microsomes from both groups of animals (K_m values were 7.1 ± 1.3 and 8.1 ± 1.4 μM for untreated and metyrapone treated rats, respectively, N = 3; Table 1). The K_m for the cofactor NAD was also unchanged by metyrapone treatment (200 ± 10 and 190 ± 30 μM for untreated and metyrapone-treated rats, respectively, N = 3; Table 1). Thus, the decrease in the rate of decanal dehydrogenation following metyrapone administration was attributed to a

FIG. 2. Immunoreactive mALDH protein in microsomes from untreated (lanes 1 and 2) and metyrapone-treated (lanes 3 and 4) female rats.

TABLE 1. Effect of metyrapone pretreatment of female rats on the kinetic parameters of mALDH-catalyzed decanal dehydrogenation in hepatic microsomes and the immunoquantitation of mALDH protein

	Decanal		NAD		
Treatment	K_m *(μM)*	V_{max} *(nmol/min/mg)*	K_m *(μM)*	V_{max} *(nmol/min/mg)*	*mALDH (ng/μg)*
None	7.1 ± 1.3	330 ± 10	200 ± 10	350 ± 20	23 ± 2
Metyrapone (100 mg/kg)	8.1 ± 1.4	200 ± 10*	190 ± 30	190 ± 20*	23 ± 2

Data are expressed as the means ± SD for N = 3 individual microsomal fractions for each group except for protein content where N = 5.
* Significantly different from the untreated group, $P < 0.001$.

decrease in the maximal reaction velocities (V_{max} values) for both decanal and NAD. These decreases in V_{max} were approximately 39 and 46%, respectively, for decanal and NAD (N = 3, $P < 0.001$). Despite these clear changes in mALDH-catalyzed decanal dehydrogenation after *in vivo* administration of metyrapone to rats, it is noteworthy that metyrapone had no direct inhibitory effect on decanal dehydrogenation *in vitro* (data not shown).

Effects of other Pyridyl Compounds on Microsomal Decanal Dehydrogenation

A series of chemicals with structural similarities to metyrapone were administered to rats to investigate their effects on microsomal decanal dehydrogenation. Most of these agents possess 3'-pyridyl systems: 2,3-di-3-pyridyl-2,3-butanediol contains two pyridyl moieties and a two-carbon chain between the pyridyl rings that is similar to the system present in metyrapone. 3-Benzoylpyridine has been shown to undergo metabolism along pathways similar to those for metyrapone [20], and since metyrapone is known to undergo *N*-oxidation [21], 3-picoline *N*-oxide was also chosen for investigation. Another analogue, α-pyridoin, is a 2'-dipyridyl compound with a two-carbon chain bridging the pyridine rings. At a dose of 100 mg/kg, none of these analogues altered microsomal decanal dehydrogenation compared with the activity in untreated microsomes (data not shown). This is in contrast to the distinct inhibitory effect displayed after metyrapone treatment at the same dose.

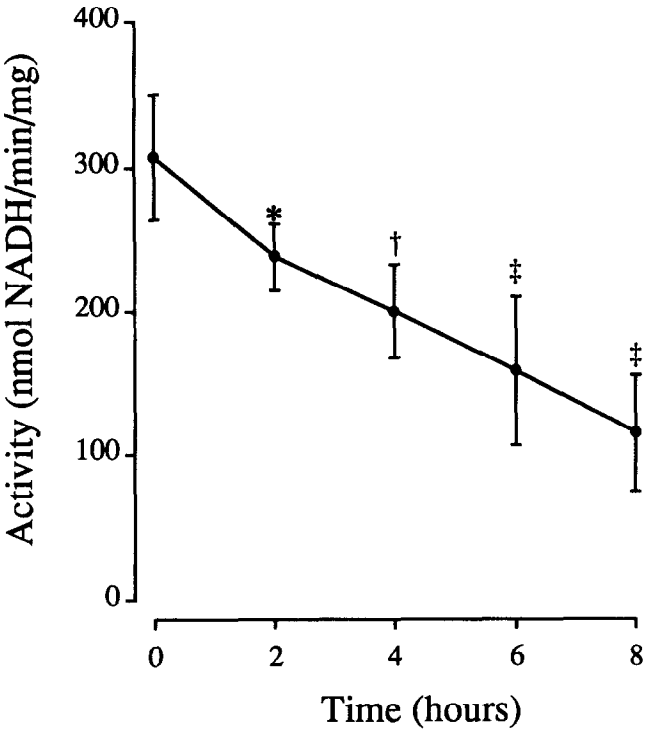


FIG. 3. Time-dependent inhibition of decanal dehydrogenation in hepatic microsomes following administration of metyrapone (100 mg/kg) to female rats. Data are expressed as means ± SD with 4 rats in each group. Significant difference from the zero time point treatment group: (*) $P < 0.05$; (†) $P < 0.005$; and (‡) $P < 0.001$.

DISCUSSION

The present study established that the *in vivo* treatment of rats with the CYP inhibitor and inducer metyrapone de-

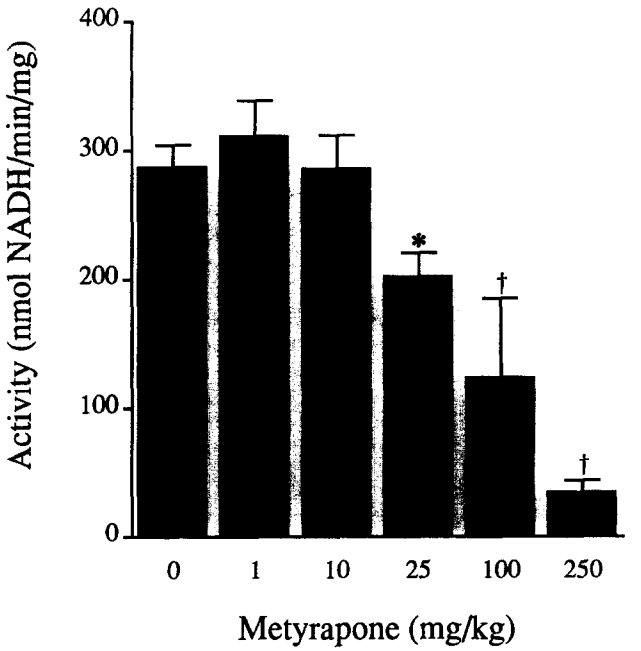
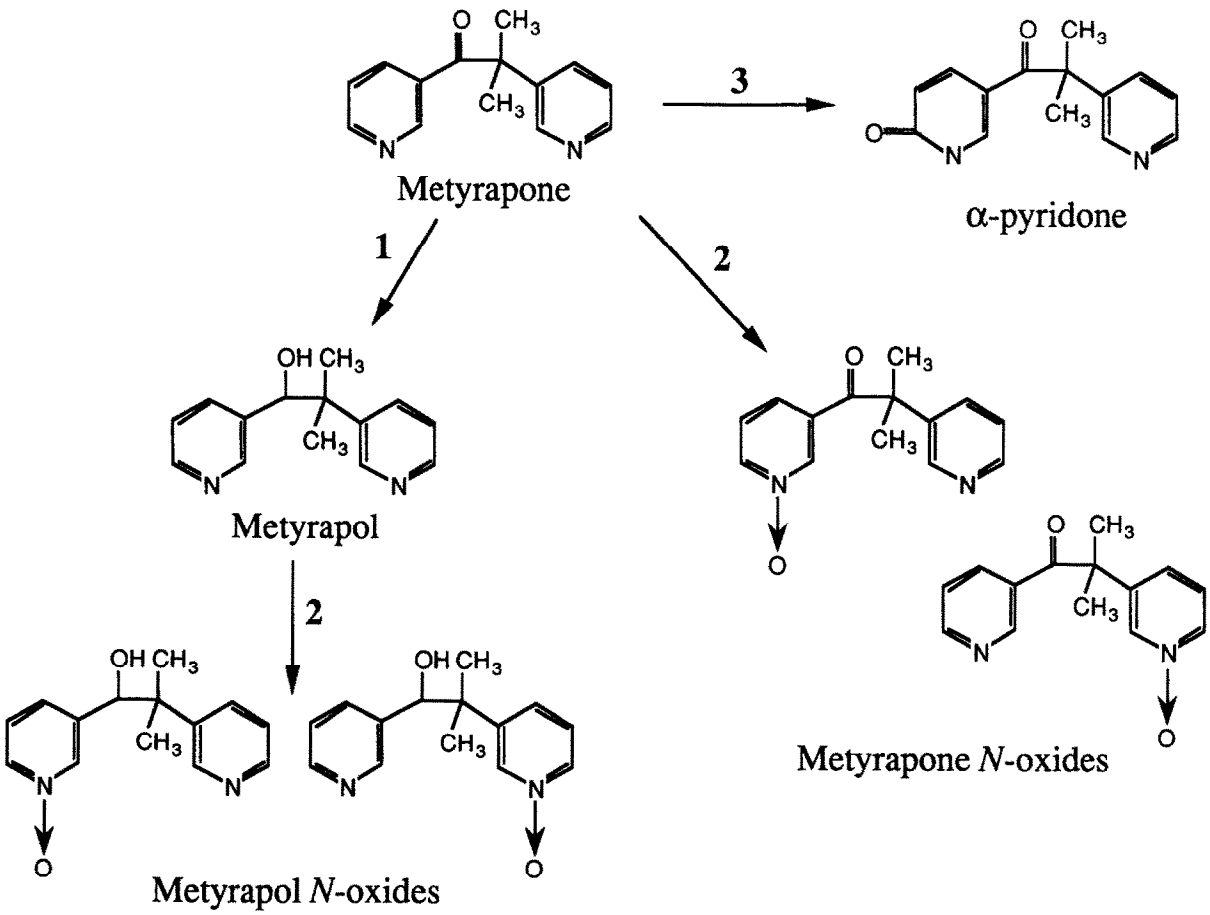


FIG. 4. Dose-dependent inhibition of decanal dehydrogenation in hepatic microsomes following administration of metyrapone to female rats. Data are expressed as means ± SD with 4 rats in each group. Significant difference from the untreated group: (*) $P < 0.005$; and (†) $P < 0.001$.

creases the rate of mALDH-catalyzed decanal dehydrogenation. This decrease in activity occurred without a change in the immunoreactive mALDH protein content—a finding that is consistent with post-translational modification of the enzyme that occurs without apparent effect on the regulation of the mALDH at other levels. Kinetic measurements support this assertion since the inhibited enzyme has Michaelis constants for decanal and NAD that are similar to those in control liver. Accordingly, the apparent affinity of the mALDH for decanal is unchanged by metyrapone administration. The only changes that were noted were decreases in the maximal reaction velocities for both decanal and NAD in microsomes from metyrapone-treated rats. This type of behavior may be expected if a portion of the mALDH was inactivated and unavailable for catalysis. This inhibitory effect of metyrapone appeared restricted to the mALDH because it was noted in the present study that cytosolic ALDH activity (using 3-hydroxybenzaldehyde as substrate) and other microsomal oxidoreductases active on steroid substrates were not inhibited after metyrapone treatment.

The precise nature of the inhibitory effect of metyrapone on mALDH activity *in vivo* remains unclear. Metyrapone is neither directly inhibitory towards microsomal decanal dehydrogenation *in vitro* nor does it act as a substrate for mALDH even though it contains a carbonyl moiety. In light of these observations and the rapid time-dependent inhibitory effect of metyrapone on decanal dehydrogenase activity, it is likely that inhibition of the mALDH may be mediated by a metabolite. At least three possible biotransformation pathways are operative for metyrapone (Scheme 1). The most prominent pathway appears to be reduction at the carbonyl group to yield metyrapol (the secondary alcohol), a reaction catalyzed by microsomal and cytosolic carbonyl reductases [22, 23]. Oxidation of the pyridyl rings catalyzed by CYP enzymes results in the formation of metyrapone *N*-oxides [21] while an unidentified cytosolic enzyme catalyzes C-oxidation to produce an α-pyridone [24]. Significantly, *in vitro* metabolism of metyrapone has been shown to be quite slow and requires high substrate concentrations [22], in contrast to the *in vivo* pharmacokinetics where a 50 mg/kg dose of metyrapone was metabolized completely within 4 hr [25]. The identification of metyrapol *N*-oxides and other unidentified metabolites following the administration of metyrapone illustrates the extensive nature of *in vivo* metabolism [26]. If a series of metabolic steps are needed in order to produce the inhibitory species, then this may not be evident *in vitro*.



SCHEME 1. Proposed pathways of metyrapone biotransformation. Enzymes catalyzing the particular reactions are denoted by the numbers: (1) microsomal/cytosolic carbonyl reductases; (2) microsomal CYP enzymes; and (3) unidentified cytosolic enzyme.

It is conceivable that the active inhibitory species would have to be formed in the vicinity of the microsomal membrane in order to interact with the mALDH. It would be anticipated that metyrapone *N*-oxides would be a possible candidate as the inhibitory species because their formation is catalyzed by membrane-bound CYP enzymes [21]. However, in experiments where metyrapone biotransformation in NADPH-supplemented microsomes *in vitro* was followed by measurement of decanal dehydrogenase activity, this did not result in mALDH inhibition. This suggests either that the concentration of the inhibitory metabolite formed is inadequate or that a different metabolite is formed *in vivo*, but not *in vitro*, and is the inhibitory species. Metyrapol *N*-oxides have been identified as the major urinary metabolites of metyrapone *in vivo* and have not been detected *in vitro*; these may be possible inhibitors of mALDH activity [26]. The pyridyl compound 3-benzoylpyridine has been shown to follow metabolic pathways similar to those of metyrapone, including carbonyl reduction and *N*-oxidation [20], but neither this compound nor 3-picoline *N*-oxide was inhibitory towards decanal dehydrogenation. This finding suggests that metyrapone *N*-oxides may, in fact, not be the inhibitory agents. In the present study, two other substituted pyridines similar in structure to metyrapone, α-pyridoin and 2,3-di-3-pyridyl-2,3-butanediol, were also without effect on decanal dehydrogenation when administered *in vivo*. Despite these findings, it remains a possibility that metyrapone elicits inhibition of mALDH-catalyzed decanal dehydrogenation because it forms an unusual reactive metabolite that is not generated from the other pyridyl derivatives *in vivo*.

The nature of the *in vivo* inhibition of mALDH activity by metyrapone is similar to that produced by disulfiram and chlorpropamide on acetaldehyde metabolism [27, 28]. Both disulfiram and chlorpropamide undergo biotransformation to a reactive metabolite that interacts with the mitochondrial ALDH and inhibits the dehydrogenation of acetaldehyde that is produced after ethanol exposure. The proposed pathways of chlorpropamide metabolism can result in the formation of either *n*-propylisocyanate or nitroxyl, and both of these agents have been shown to be inhibitors of mitochondrial ALDH [28]. Similarly, other *N*-substituted chlorpropamide derivatives promote the release of the inhibitory species, but it is still unclear which of the pathways of chlorpropamide metabolism occur *in vivo* [29]. Furthermore, *in vivo* inhibition of acetaldehyde metabolism by *n*-butyraldoxime requires bioactivation of this chemical by CYP enzymes for inhibition to occur. Although 1-nitrobutane and butyronitrile were identified as products of CYP-mediated metabolism, neither of these agents was inhibitory *in vitro* so that inhibition may be mediated by an unidentified metabolite [30].

In summary, the data presented suggest strongly that the rat hepatic mALDH is not as responsive to xenobiotics as CYP enzymes and other hepatic genes. Metyrapone is one of the few agents that has been shown to alter significantly the activity of mALDH. It is now of considerable interest to determine the nature of the metyrapone-derived species that is involved in mALDH inactivation. Such information would be of value in assessing the structural requirements for modulation of mALDH by xenobiotics.

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