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ROLE OF FLAVIN-DEPENDENT MONOOXYGENASES AND CYTOCHROME P450 ENZYMES IN THE SULFOXIDATION OF *S*-METHYL *N,N*-DIETHYLTHIOLCARBAMATE

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Abstract—Disulfiram is bioactivated to *S*-methyl *N,N*-diethylthiolcarbamate sulfoxide (DETC-MeSO), the metabolite proposed to be responsible for the action of disulfiram as an aldehyde dehydrogenase inhibitor. This bioactivation process includes a reduction, an *S*-methylation, and two successive oxidations. Sulfur-containing functional groups are substrates for cytochrome P450 enzymes or flavin-containing monooxygenases (FMO). In the present study, we investigated the contribution of these monooxygenases to the formation of DETC-MeSO from its immediate precursor *S*-methyl *N,N*-diethylthiolcarbamate (DETC-Me). Liver microsomes obtained from mature male rats were incubated with DETC-Me. The formation of DETC-MeSO was blocked completely by solubilization of the microsomes with the detergent Emulgen 911, or by the presence of the cytochrome P450 inhibitor 1-benzylimidazole. However, thermal-inactivation of FMO resulted in only a partial loss in DETC-MeSO formation. Liver microsomes from phenobarbital-treated rats showed a 4- to 5-fold increase in the rate of formation of DETC-MeSO, compared with controls. Liver microsomes from pyrazole-treated rats showed a 50% decrease in the sulfoxidation of DETC-Me compared with controls. In a purified reconstituted system, cytochrome P450 2B1 (CYP2B1) catalyzed the formation of DETC-MeSO at a rate of 51 nmol DETC-MeSO formed/min/nmol cytochrome P450. Antibodies to CYP2B1 caused a 60% inhibition of DETC-MeSO formation by liver microsomes from phenobarbital-treated rats. These results suggest that in male rat liver microsomes, cytochrome P450 plays a major role in catalyzing the sulfoxidation of DETC-Me, whereas FMO plays a minor role (<10%). Also, in liver microsomes from phenobarbital-treated rats, CYP2B1 is the major catalyst for the sulfoxidation of DETC-Me.

Disulfiram has been used as an alcohol deterrent agent for the treatment of alcoholism for almost 50 years [1]. However, it is only recently that Yourick and Faiman [2] established the importance of bioactivation in the action of disulfiram as a liver aldehyde dehydrogenase (ALDH)§ inhibitor. The steps involved in the bioactivation of disulfiram are described by Hart and Faiman [3].

The proposed terminal step in the bioactivation of disulfiram is the sulfoxidation of *S*-methyl *N,N*-diethylthiolcarbamate (DETC-Me) to *S*-methyl *N,N*-diethylthiolcarbamate sulfoxide (DETC-MeSO) [4], a reaction blocked *in vivo* by the cytochrome P450 inhibitor 1-octylimidazole (*N*-octylimidazole; NOI) [2]. NADPH-dependent oxygenations in liver microsomes are mediated through a cytochrome P450-dependent monoxygenase or flavin-containing monooxygenase (FMO). Virtually all sulfur-containing functional groups are substrates for oxidation by cytochrome P450 and FMO [5, 6]. The findings by Yourick and Faiman [2] that prior administration of the nonspecific cytochrome P450 inhibitor NOI to rats blocks rat liver mitochondrial low K_m ALDH inhibition by disulfiram, diethyldithiocarbamate (DDTC), diethyldithiocarbamate-methyl ester (DDTC-Me), and DETC-Me suggested a role for cytochrome P450. However, it is also possible that the oxidative attack on DETC-Me may occur by an FMO-mediated mechanism which would be consistent with the FMO-catalyzed oxygenation of *S*-alkylthiocarbamates [7]. Thus, either or both monooxygenases may be important in disulfiram bioactivation. In the present studies, the relative role of FMO and cytochrome P450 enzymes in the sulfoxidation of DETC-Me to DETC-MeSO was investigated. The findings from these studies suggest that cytochrome P450 plays a major role in catalyzing the sulfoxidation of DETC-Me to DETC-MeSO, with only a minor contribution from FMO. Furthermore, in liver microsomes from phenobarbital-treated rats, cytochrome P450 2B1 (CYP2B1) appears to be the major cytochrome P450 isozyme responsible for the sulfoxidation of DETC-Me.

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§ Abbreviations: ALDH, aldehyde dehydrogenase; DDTC, diethyldithiocarbamate; DDTC-Me, diethyldithiocarbamate-methyl ester; DETC-Me, *S*-methyl *N,N*-diethylthiolcarbamate; DETC-MeSO, *S*-methyl *N,N*-diethylthiolcarbamate sulfoxide; NOI, 1-octylimidazole; and FMO, flavin-containing monooxygenase(s).

MATERIALS AND METHODS

Materials. DETC-Me [8] and DETC-MeSO [4]

were synthesized as described previously. Molinate sulfoxide, employed as an internal standard, was synthesized by the method of Casida *et al.* [9]. Phenobarbital sodium, 1-benzylimidazole and Emulgen 911 (Igepal CO-720) were obtained from the Aldrich Chemical Co., Inc. (Milwaukee, WI). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). C_{18} Bond-elut extraction columns were purchased from Varian Sample Preparation Products (Harbor City, CA).

Animals. Sprague–Dawley derived male rats (7 to 8-weeks-old) were used for the preparation of liver microsomes. The animals were bred from a resident colony maintained in the Animal Care Unit of The University of Kansas. Rats were maintained on a 12-hr light–dark cycle with access to Purina rat chow and water *ad lib.* The night before the preparation of microsomes, food, but not water was removed from the animal cages.

Drug administration. Sodium phenobarbital (80 mg/kg in saline) was administered i.p. daily for 3 days. Pyrazole (200 mg/kg in saline) was administered i.p. daily for 2 days.

Preparation of microsomes. Microsomes were prepared from untreated (controls), phenobarbital-treated, or pyrazole-treated mature male rats (7 to 8-weeks-old). The rats were anesthetized with CO_2 and decapitated. The livers were removed and placed in potassium phosphate buffer (0.1 M, pH 7.4) containing 1.12% (w/v) KCl and 1 mM EDTA at 4°. The livers were then minced and homogenized. The homogenate was subjected to centrifugation for 15 min at 3,000 *g*, and the resulting supernatant was subjected to centrifugation for 20 min at 12,000 *g*. The pellet was discarded, and the supernatant was then subjected to centrifugation for 60 min at 105,000 *g*. The microsomal pellet was washed once and resuspended in 0.1 M, pH 7.4, potassium phosphate buffer without KCl. Protein content was determined by the method of Lowry *et al.* [10] using bovine serum albumin as the standard. Microsomes were frozen and stored at −70° until used.

Purification of NADPH–cytochrome P450 reductase and CYP 2B1. NADPH–cytochrome P450 reductase was purified by a modification of the method described by Yasukochi and Masters [11], and its concentration was determined as described previously [12]. CYP2B1 was purified as described previously [13].

Preparation and purification of antibodies against CYP2B1. Antibodies against CYP2B1 were raised in male New Zealand White rabbits and purified, as described previously [14, 15].

Enzyme assays. Total cytochrome P450 content of the liver microsomes was determined by the method of Omura and Sato [16] from the CO-difference spectrum of dithionite-reduced microsomes, based on an extinction coefficient of 91 $mM^{-1} cm^{-1}$.

Benzphetamine demethylase activity was determined by incubating benzphetamine-HCl microsomes obtained from mature untreated male rats. The incubations were carried out for 10 min at 37°. The volume of the incubation mixture was 1.5 mL, and contained potassium phosphate buffer (0.1 M, pH 7.4), EDTA (1 mM), $NADP^+$ (0.67 mM), glucose-6-phosphate (6.67 mM), glucose-6-phosphate dehydrogenase (0.67 U/mL), semicarbazide (5 mM), control microsomes (1.0 mg/mL), and benzphetamine (0.7 mM) with or without other additions as described in the Results. The reaction was initiated by the addition of benzphetamine in 42 μL of phosphate buffer, and the incubation was carried out in a shaking metabolic incubator. The reaction was stopped by the addition of 0.4 mL of 15% (w/v) $ZnSO_4$ followed by 0.4 mL of saturated $Ba(OH)_2$. The precipitate was removed by centrifugation, and 1 mL of the supernatant fraction was analyzed for formaldehyde by the method of Nash [17].

Thiobenzamide *S*-oxidation was determined by incubating thiobenzamide in the presence of control microsomes (from mature untreated male rats) employing the method of Cashman and Hanzlik [18]. Incubations were carried out at 35° in 4.5 mL-1 cm path length cuvets, and the reaction was monitored for 5 min in a Shimadzu UV-160 at 370 nm. The cuvets were maintained for 5 min in the spectrophotometer before the addition of thiobenzamide contained in 42 μL acetonitrile. A lag time of 2 min was maintained between the addition of thiobenzamide and observing the rate of change in absorbance. This procedure was used to stabilize the temperature in the cuvet. The reaction was carried out in a 3.5-mL incubation that included potassium phosphate buffer (0.1 M, pH 7.4), EDTA (1 mM), control microsomes (1.5 mg protein), $NADP^+$ (0.67 mM), glucose-6-phosphate (6.67 mM) glucose-6-phosphate dehydrogenase (0.67 U/mL), and thiobenzamide (1 mM) with or without other additions as described in the Results.

Thermal-inactivation of microsomal FMO. The thermal-inactivation of microsomal FMO was carried out by the method of Poulsen *et al.* [19]. A concentrated suspension of microsomes (10 mg/mL) in 0.1 M potassium phosphate buffer (pH 7.4) containing 200 μM butylated hydroxytoluene (BHT) was heated rapidly to 50° and maintained at 50° for 1 min after which the tube was immediately chilled in ice. All experiments using heat-inactivated microsomes were carried out using the same microsomal suspension on the same day.

Sulfoxidation of DETC-Me. Sulfoxidation of DETC-Me to DETC-MeSO was carried out in 1.5-mL incubation mixtures that included potassium phosphate buffer (0.1 M, pH 7.4), EDTA (1 mM), control microsomes (0.67 mg/mL protein), $NADP^+$ (0.67 mM), glucose-6-phosphate (6.67 mM) and glucose-6-phosphate dehydrogenase (0.67 U/mL) with or without other additions as described in the Results. The reactions were initiated by the addition of DETC-Me in 15 μL acetonitrile (final concentration 1 mM), and the incubation was carried out in a shaking metabolic incubator. The reactions were carried out for 5, 10, 15, 20, 30, 45, and 60 min. After each incubation for the particular time period, the reaction was terminated by the addition of $CaCl_2$ (final concentration 20 mM). Time zero was determined by the addition of $CaCl_2$ immediately after the addition of the substrate. Usual techniques, such as acid or base precipitation, were not used for terminating the reaction because the acid or base

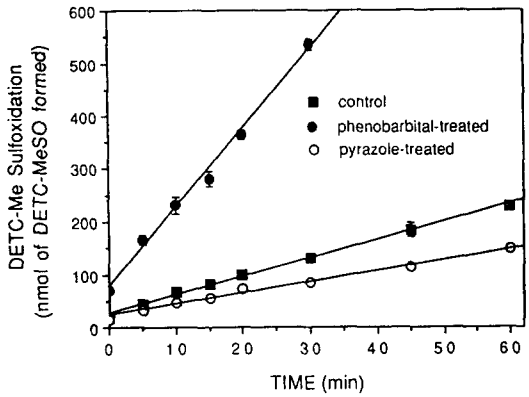

Fig. 1. Rate of formation of DETC-MeSO in microsomal incubations. Liver microsomes were obtained from untreated, phenobarbital-treated, and pyrazole-treated male rats. Microsomes (1 mg protein) were incubated at 37° with DETC-Me (1 mM) and an NADPH-generating system, the reaction was terminated at the times indicated, and the amount of DETC-MeSO formed was determined by HPLC as described under Materials and Methods. Each point is the mean ± SEM for four determinations. The data are corrected for the extraction efficiency of DETC-MeSO.

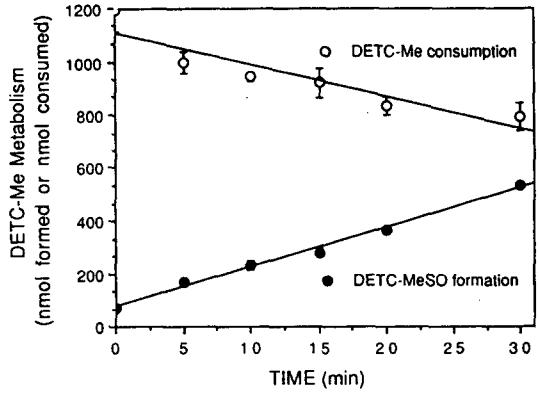

Fig. 2. Mass balance for the formation of DETC-MeSO and the loss of DETC-Me in a microsomal incubation. Liver microsomes were obtained from phenobarbital-treated male rats. Microsomes (1 mg protein) were incubated at 37° with DETC-Me (1 mM) and an NADPH-generating system, the reaction was terminated at the times indicated, and the amount of DETC-MeSO formed and the amount of DETC-Me consumed were determined by HPLC as described under Materials and Methods. Each point is the mean ± SEM for four determinations. The data are corrected for the extraction efficiency of DETC-Me and DETC-MeSO.

stability of DETC-MeSO is unknown at this time. Precipitated protein was removed by centrifugation and the supernatant removed. Molinate sulfoxide (10 μL of 10 mM stock in acetonitrile) was added to 1.0 mL of the supernatant, and the supernatant was loaded on a 500 mg (3 mL) C_{18} Bond-elut column that had been washed and equilibrated with 3 mL methanol and potassium phosphate buffer (0.1 M, pH 7.4), respectively. The columns were washed with 5 mL distilled water and then eluted with 1 mL acetonitrile. The samples were then analyzed by HPLC at room temperature. An Alltech cyano column (250 × 4.6 mm, 5 μm particle size), an Altex/Beckman solvent pump (0.8 mL/min), a 20-μL sample loop and a UV detector (215 nm) were used for HPLC analysis. The extraction efficiencies based on measurements with spiked samples were 74, 72, and 100% for DETC-MeSO, molinate sulfoxide and DETC-Me, respectively. The detector response was linear in the concentration range studied.

Antibody inhibition studies. The conditions for the antibody inhibition studies were as those described above except for the following modifications. The amount of microsomal protein was reduced to 0.05 mg, and the microsomes were preincubated with various concentrations of rabbit anti-rat CYP2B1 for 5 min; then the NADPH-generating system and the substrate were added. The concentration of antibody was adjusted to 0.67 mg/mL with an IgG fraction purified from rabbit preimmune serum. A 100 mg (1 mL) C_{18} Bond-elut column was used for all extractions, and the wash and final elution were carried out with 0.3 mL distilled water and 0.3 mL acetonitrile (33%, v/v), respectively. The extraction efficiencies based on measurement with spiked samples were 43 and 100% for DETC-MeSO and molinate sulfoxide, respectively. The detector response was linear in the concentration range studied.

Purified CYP2B1 reconstitution studies. Purified CYP2B1 was reconstituted as described above except for the following modifications. The concentration of lipid (dilauroyl phosphatidylcholine) was optimized to 20 μM. Purified rat liver CYP2B1 (0.02 nmol), purified NADPH–cytochrome P450 reductase (various concentrations), and 20 μM lipid (stock of 1 mg/mL was sonicated for 2 min just before the incubation) were mixed in 250 μL, pH 7.4, 0.1 M potassium phosphate buffer. The NADPH-generating system (see above) and the buffer were added giving a final volume of 1.5 mL. The reaction was initiated by the addition of DETC-Me (final concentration 1 mM). The reaction was incubated for 60 min and then terminated by the addition of 1 mM 1-benzylimidazole. The extraction efficiencies based on measurements with spiked samples were 36 and 100% for DETC-MeSO and molinate sulfoxide, respectively. The detector response was linear in the concentration range studied.

RESULTS

Rate of formation of DETC-MeSO in microsomal incubation. Phenobarbital and pyrazole are classical inducers of CYP2B1 and CYP2E1 enzymes in the liver, respectively. Liver microsomes obtained from untreated, phenobarbital-treated, and pyrazole-treated mature male rats were incubated with DETC-Me. Formation of DETC-MeSO was linear with time in all groups of microsomes (Fig. 1). The intercept is not zero, and reflects the time-lag

Table 1. Rate of formation of DETC-MeSO in rat microsomal incubation

Source of rat liver microsomes	Cytochrome P450 (nmol/mg protein)	DETC-MeSO formation (nmol formed/ min/mg protein)	DETC-MeSO formation (nmol formed/min/ nmol cytochrome P450)
Untreated	0.64 ± 0.03	3.8 ± 0.1	5.9 ± 0.2
Phenobarbital-treated	1.58 ± 0.01	17.8 ± 0.3	11.2 ± 0.2
Pyrazole-treated	1.04 ± 0.01	2.5 ± 0.1	2.4 ± 0.04

Cytochrome P450 content of microsomes was determined by a CO-difference spectrum of dithionite-reduced proteins. Liver microsomes were obtained from untreated, phenobarbital-treated, and pyrazole-treated male rats. Microsomes (1 mg protein) were incubated at 37° with DETC-Me (1 mM) and an NADPH-generating system. The reaction was terminated at various times, and the amount of DETC-MeSO formed was determined by HPLC as described under Materials and Methods. Values are the means ± SEM for four determinations. The data are corrected for the extraction efficiency of DETC-MeSO.

Table 2. Relative contribution of cytochrome P450 and FMO in the sulfoxidation of DETC-Me

	DETC-Me sulfoxidation	Benzphetamine demethylation	Thiobenzamide *S*-oxidation
	(nmol of product formed/min/mg protein)		
Control	5.8 ± 0.2 (100)	2.8 ± 0.4 (100)	11.9 ± 0.6 (100)
Control + Emulgen 911	0.3 ± 0.01* (6)	0.7 ± 0.07* (24)	11.4 ± 0.1 (96)
Control + 1-benzylimidazole	0.6 ± 0.02* (10)	0.1 ± 0.06* (4)	5.0 ± 0.2* (42)
Control (heat-inactivation)	3.2 ± 0.04* (56)	1.7 ± 0.06*† (78)	2.0 ± 0.1* (17)

The same batch of control microsomes obtained from untreated rats was used for these experiments. DETC-Me sulfoxidation, benzphetamine *N*-demethylation, and thiobenzamide *S*-oxidation assays were carried out as described under Materials and Methods. The detergent [final concentration, 1% (w/v)] used was Emulgen 911. The final concentration of 1-benzylimidazole used was 1 mM. The heat-inactivation of microsomes is described under Materials and Methods. Values given in parentheses represent percent of control. Values are the means ± SEM for four determinations.

* Bonferroni's P value < 0.05 when compared with the control group, as determined by a one-way analysis of variance.

† The control value for this experiment was 2.2 ± 0.05.

between the addition of substrate and the termination of the reaction. Microsomes from phenobarbital-treated rats exhibited approximately a 4-fold increase in reaction rate, compared with microsomes from untreated rats, whereas approximately 50% inhibition was observed with microsomes from pyrazole-treated rats (Table 1).

The rate of formation of DETC-MeSO and the rate of loss of DETC-Me in a typical microsomal incubation were determined. The slopes of the two lines were similar and opposite in sign, which suggests that in a rat liver microsomal incubation containing DETC-Me, the major product formed is DETC-MeSO (Fig. 2).

Relative role of cytochrome P450 and FMO in sulfoxidation of DETC-Me. DETC-Me sulfoxidation was induced 4- to 5-fold by phenobarbital, which suggested at least partial involvement of cytochrome P450 (Table 1). Thiobenzamide and benzphetamine are classical FMO and cytochrome P450 substrates, respectively [18, 20]. Using untreated male rat liver microsomes, thiobenzamide *S*-oxidation has been shown to be approximately 65% FMO-catalyzed and 35% cytochrome P450-catalyzed [21]. Therefore, benzphetamine demethylation and thiobenzamide *S*-oxidation were carried out with control microsomes with or without the addition of inhibitor in order to investigate the relative role of cytochrome P450 and FMO (Table 2). Uninhibited rates of benzphetamine demethylation and thiobenzamide *S*-oxidation were similar to those previously reported [20, 21]. The addition of non-ionic detergent (Emulgen 911) to the microsomal incubation produced an almost total loss in DETC-MeSO formation, a 75% decrease in benzphetamine demethylation, and had no effect on thiobenzamide *S*-oxidation. When the cytochrome P450 inhibitor 1-benzylimidazole [22] was included in the incubation, thiobenzamide *S*-oxidation was inhibited by 60%, whereas DETC-MeSO formation and benzphetamine demethylation were inhibited completely (Table 2). Thermal-inactivation of FMO activity in microsomes resulted in 40, 20 and 80% decrease in DETC-MeSO formation, benzphetamine demethylation, and thiobenzamide *S*-oxidation, respectively (Table 2).

Purified reconstituted cytochrome P450 studies. Sulfoxidation of DETC-Me was studied in a purified CYP2B1 reconstituted system. The data obtained

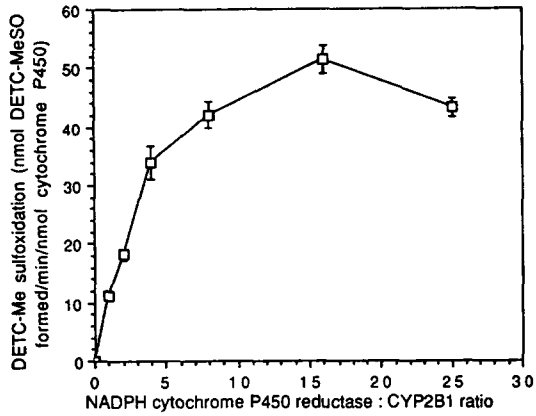

Fig. 3. Effect of changing the NADPH–cytochrome P450 reductase to CYP2B1 ratio on the rate of formation of DETC-MeSO from DETC-Me. The optimum concentration of dilauroyl phosphatidylcholine was determined to be 20 μM. The mixtures, containing 0.02 nmol of purified CYP2B1, 20 μM dilauroyl phosphatidylcholine, DETC-Me (1 mM), NADPH-generating system, and various concentrations of NADPH–cytochrome P450 reductase (0.02, 0.04, 0.08, 0.16, 0.32 and 0.48 nmol), were incubated at 37° for 60 min. The amount of DETC-MeSO formed was determined by the HPLC method described under Materials and Methods. Each point is the mean ± SEM for four determinations. The data are corrected for the extraction efficiency of DETC-MeSO.

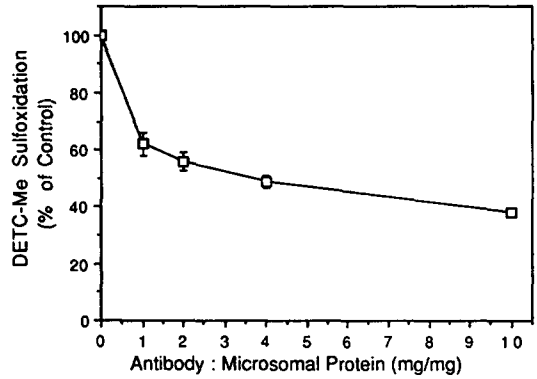

Fig. 4. Inhibition of the formation of DETC-MeSO by anti-CYP2B1. Microsomal protein from phenobarbital-treated male rat liver (0.05 mg) was mixed with various amounts of purified rabbit anti-rat CYP2B1 (0.0, 0.05, 0.1, 0.2, and 0.5 mg). The total IgG was then made up to 1 mg using IgG obtained from preimmune serum. The above mixture was incubated for 5 min at 37°, and the tubes were chilled in ice. DETC-Me (1 mM) and an NADPH-generating system were added, and the incubation was carried out for 60 min at 37°. The reaction was terminated, and the amount of DETC-MeSO formed was determined by an HPLC method described under Materials and Methods. The control value for 0.0 mg antibody was found to be 13.0 ± 0.24 nmol of DETC-MeSO formed/min/nmol cytochrome P450. Each point is the mean ± SEM for four determinations. The data are corrected for the extraction efficiency of DETC-MeSO.

from incubation of various microsomes with DETC-Me (Table 1) suggested that phenobarbital-inducible cytochrome P450 enzymes are good catalysts for DETC-Me sulfoxidation. Thus, the major phenobarbital-inducible enzyme, CYP2B1, was selected for these reconstitution studies. When purified CYP2B1 was incubated with an optimum amount of lipid, NADPH-generating system and saturating amounts of NADPH cytochrome P450 reductase, a turnover number of 51 min^{-1} was obtained (Fig. 3). This rate is 4–5 times the rate of DETC-Me sulfoxidation by liver microsomes from phenobarbital-treated rats (11.2 min^{-1}) (Table 1).

Antibody inhibition studies. The sulfoxidation of DETC-Me in microsomes from phenobarbital-treated rats was examined in the presence of antibodies against CYP2B1. The polyclonal antibody to CYP2B1 has been shown previously to inhibit CYP2B1-dependent metabolism in microsomal incubations [14]. The effect of increasing antibody concentration on DETC-Me sulfoxidation by liver microsomes from phenobarbital-treated rats is shown in Fig. 4. DETC-Me sulfoxidation was inhibited 60% when a 10-fold excess of antibody was used.

DISCUSSION

Although disulfiram has been used as an alcohol deterrent for almost 50 years [1], its efficacy remains uncertain. For example, there have been continued reports of disulfiram-treated individuals not experiencing a disulfiram–ethanol reaction (DER) after an ethanol challenge [23], or disulfiram having limited effectiveness in promoting abstinence [24]. Guengerich and Turvy [25] have shown that the cytochrome P450 enzymes CYP1A1 and CYP2E1 are depressed in hepatic diseases such as cirrhosis. Since disulfiram is used in the treatment of chronic alcohol abuse, bioactivation of disulfiram may be impaired in these patients, thus providing one explanation for the ineffectiveness of disulfiram in some alcoholics.

The importance of disulfiram bioactivation [2] and the identification of DETC-MeSO as the chemical species responsible for the action of disulfiram as an ALDH inhibitor [4] have been recognized only recently. The proposed terminal step in the bioactivation of disulfiram is the sulfoxidation of DETC-Me to DETC-MeSO [4]. Employing relatively specific substrates such as benzphetamine and thiobenzamide for cytochrome P450 and FMO, respectively, the effects of detergent (Emulgen 911), 1-benzylimidazole, and thermal inactivation on cytochrome P450 activity, FMO activity and sulfoxidation of DETC-Me were determined. Addition of Emulgen 911 to the microsomal incubation has been shown to inhibit cytochrome P450 [26]. Similar results were obtained when the benzphetamine-demethylase assay, which reflects cytochrome P450 activity, was carried out in the presence of Emulgen 911. In contrast to cytochrome P450, FMO activity increased in the presence of Emulgen 911 (Table 2). Since thiobenzamide *S*-oxidation is catalyzed partly by FMO and partly by cytochrome P450 [26], no net increase in the rate of thiobenzamide *S*-oxidation was observed in the

presence of Emulgen 911 (Table 2). DETC-MeSO formation was inhibited almost completely when Emulgen 911 was included in the microsomal incubation (Table 2). When the cytochrome P450 inhibitor, 1-benzylimidazole, was included in the incubation, benzphetamine demethylation and DETC-Me sulfoxidation were both inhibited more than 90%. Thiobenzamide *S*-oxidation was also partially inhibited by 1-benzylimidazole, which is to be expected since thiobenzamide *S*-oxidation in rat liver microsomes is catalyzed partly by cytochrome P450 and partly by FMO [21]. Thermal-inactivation of FMO was reflected by the reduced rate of thiobenzamide *S*-oxidation, whereas the benzphetamine demethylation and DETC-Me sulfoxidation were inhibited to a lesser degree (Table 2). It should be noted that cytochrome P450 was also partially inactivated by thermal-inactivation. These results suggest that at least 90% of DETC-Me sulfoxidation observed in microsomes from untreated rats is catalyzed by cytochrome P450 and that FMO has little, if any, role to play in the formation of DETC-MeSO. Since it is known that the specific activity of FMO is much lower in human liver compared with rat liver [27], the contribution of FMO in catalyzing the sulfoxidation of DETC-Me in humans may be negligible.

Studies employing liver microsomes obtained from either untreated, phenobarbital-treated, or pyrazole-treated rats were carried out. A 4-fold increase in the rate of formation of DETC-MeSO was observed with microsomes from phenobarbital-treated rats, whereas an approximately 50% decrease was observed with microsomes from pyrazole-treated rats when compared with untreated rats (Fig. 1; Table 1). This suggests that phenobarbital-inducible enzymes may play a role in catalyzing the sulfoxidation of DETC-Me. Since the specific activity of phenobarbital-inducible cytochrome P450 enzymes in microsomes from untreated rats is very low [28], more than one enzyme must play a role in DETC-MeSO formation when microsomes from untreated rats are used. Pryazole induction increases CYP2E1 levels in rat liver. Although a lower reaction rate was observed when microsomes from pyrazole-treated rats were used, it is difficult to interpret this result because CYP2E1 is inhibited by organic solvents, e.g. acetonitrile, present in these incubations (Table 1). CYP2E1 is induced by ethanol [29], but the available data in these studies do not allow any predictions to be made regarding the correlation of bioactivation and efficacy of disulfiram in alcoholics.

Microsomes from phenobarbital-treated rats gave the highest increase in the rate of formation of DETC-MeSO. Therefore, CYP2B1, the major phenobarbital-inducible cytochrome P450 enzyme, was selected for further studies. CYP2B1 has been shown to increase more than 25-fold in rats treated with phenobarbital [30]. With saturating amounts of NADPH–cytochrome P450 reductase and optimal amounts of lipid, the turnover number of CYP2B1 for DETC-Me sulfoxidation formation was found to be 51 min^{-1} (Fig. 3). This turnover number is about 4–5 times higher than that observed with microsomes from phenobarbital-treated rats (Table 1). The higher turnover number for the purified enzyme implicates CYP2B1 as a major catalyst of DETC-Me sulfoxidation by liver microsomes from phenobarbital-treated rats.

Polyclonal antibodies to rat CYP2B1 were preincubated with rat liver microsomes from phenobarbital-treated rats, and DETC-Me sulfoxidation was studied (Fig. 4). As the antibody to microsomal protein ratio was increased, there was a decrease in the rate of DETC-MeSO formation. A maximal inhibition of 60% was observed when the antibody to microsomal protein ratio was increased to 10. CYP2B1 and CYP2B2 share 97% amino acid sequence identity and thus cannot be distinguished with polyclonal antibodies [31]. Therefore, these results indicate that, in microsomes from phenobarbital-treated rats, at least 60% of DETC-Me sulfoxidation was catalyzed by CYP2B1 and CYP2B2. These results combined with the purified CYP2B1 reconstitution studies suggest that in liver microsomes from phenobarbital-treated male rats CYP2B1 is the major catalyst for DETC-Me sulfoxidation.

In conclusion, these studies represent the first evidence for the important role of cytochrome P450 in the formation of DETC-MeSO, the proposed active metabolite of disulfiram. Using classical P450-inducers, we have determined that treatment of rats with phenobarbital increases the rate of sulfoxidation of DETC-Me. Among the phenobarbital-inducible enzymes, CYP2B1 appears to play a major role in this reaction. Oxidative metabolism of disulfiram also occurs when DDTC-Me is desulfurated to DETC-Me. The role of cytochrome P450 and FMO in the oxidative desulfuration of DDTC-Me is unknown at this time. In addition to *S*-methylation of DDTC to DDTC-Me, these enzymatic processes are all under genetic control. This may provide one explanation for the variability in the efficacy of disulfiram between patients in reaction to an ethanol challenge [23] and thus provide a rationale for the clinical use of DETC-MeSO rather than disulfiram itself. The present study and further studies in progress should help identify the enzymes involved in disulfiram bioactivation.

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