IDENTIFICATION OF THE HUMAN AND RAT P450 ENZYMES RESPONSIBLE FOR THE 
SULFOXIDATION OF S-METHYL N,N-DIETHYLTHIOLCARBAMATE (DETC-ME) 
The Terminal Step in the Bioactivation of Disulfiram 

AJAY MADAN, ANDREW PARKINSON, AND MORRIS D. FAIMAN 
Department of Pharmacology and Toxicology (A.M., M.D.F.), University of Kansas; and Department of Pharmacology, Toxicology, and Therapeutics (A.P.), Center for Environmental and Occupational Health, University of Kansas Medical Center 

(Received April 10, 1995; accepted July 10, 1995)

ABSTRACT: 
The present study investigated the role of rat and human cytochrome P450 enzymes in the sulfoxidation of S-methyl N,N-diethyldithiocarbamate (DETC-Me) to S-methyl N,N-diethyldithiocarbamate sulfoxide (DETC-Me sulfoxide), the putative active metabolite of disulfiram. DETC-Me sulfoxidation by microsomes from male and female rats treated with various cytochrome P450-enzyme inducers suggested that multiple enzymes can catalyze this reaction, and these include, CYPIA1/2, CYP2B1/2, and CYP3A1/2. All cDNA-expressed human cytochrome P450 enzymes examined catalyzed the sulfoxidation of DETC-Me. The turnover rates (min⁻¹) of DETC-Me sulfoxidation by the cDNA-expressed cytochrome P450 enzymes ranked as follows: CYP3A4 > CYP2A6 = CYP2C9 > CYP1A2 > CYP2B6 = CYP2E1 > CYP1A1 > CYP2D6. Interestingly, CYP3A4 ranked first or last, depending on whether or not additional NADPH-cytochrome P450 reductase was coexpressed in the lymphoblastoid cells. This complicated estimates of the contribution of CYP3A4 to DETC-Me sulfoxidation by human liver microsomes. The sample-to-sample variation in DETC-Me sulfoxidation by a bank of human liver microsomes (N = 13) correlated highly with coumarin 7-hydroxylation (r = 0.88) and testosterone 6β-hydroxylation (r = 0.90), suggesting that CYP2A6 and CYP3A4/5 contribute to the sulfoxidation of DETC-Me by human liver microsomes. Although, chlorzoxazone 6-hydroxylation (a marker for CYP2E1) correlated poorly with DETC-Me sulfoxidation, the correlation improved from r = 0.07 to r = 0.44 when DETC-Me sulfoxidation was studied in the presence of the CYP2A6 inhibitor, coumarin. Similarly, when DETC-Me sulfoxidation was studied in the presence of diethyldithiocarbamate (DDTC), the inhibited DETC-Me sulfoxidase activity correlated better (r = 0.50) with chlorzoxazone 6-hydroxylase, compared with DETC-Me sulfoxidase activity in the absence of DDTC (r = 0.09). Polyclonal antibodies against CYP2E1 caused a modest inhibition (30%) of DETC-Me sulfoxidation by human liver microsomes. Anti-CYP3A1 antibodies completely inhibited DETC-Me sulfoxidation by cDNA-expressed CYP3A4. Under similar conditions, DETC-Me sulfoxidation by human liver microsomes was only partially inhibited by anti-CYP3A1 antibodies. Although studies with the rat and cDNA-expressed cytochrome P450 enzymes suggested that CYP1A2 contributed to DETC-Me sulfoxidation, this reaction was not inhibited by either furafylline (a mechanism-based inhibitor of CYP1A2) or antibodies against CYP1A1/2. A significant role for CYP2C9 was excluded by the inability of sulfaphenazole to inhibit the sulfoxidation of DETC-Me by human liver microsomes. Collectively, these data suggest that multiple cytochrome P450 enzymes can catalyze the sulfoxidation of DETC-Me. In human liver microsomes the CYP2A6, CYP2E1, and CYP3A4/5 all contribute significantly to the sulfoxidation of DETC-Me. It is interesting to note that DDTC, the reduced metabolite of disulfiram, is known to inhibit these same enzymes. The ability of DDTC to block the formation of DETC-Me sulfoxide may explain why the dose of disulfiram required to produce a disulfiram-ethanol reaction in alcoholics is so variable and often inadequate. 

The pharmacological basis for the use of disulfiram as an aversive agent or deterrent for the treatment of alcohol abuse is its inhibition of liver ALDH² (E.C. 1.2.1.3) (1). A number of studies in rats, both in vitro and in vivo, have now convincingly demonstrated that ALDH inhibition requires disulfiram bioactivation (2-9), and that the active metabolite of disulfiram is proposed to be DETC-Me sulfoxide. Although DETC-Me sulfoxide is a potential metabolite of DETC-Me sulfoxide (10, 11), DETC-Me sulfoxide is not only less potent an ALDH inhibitor than DETC-Me sulfoxide, but also is extremely toxic (10). The key sequential metabolic steps in disulfiram bioactivation are given in fig. 1. Disulfiram initially undergoes a thiol-dependent reduction (12) to DDTC. This is followed by a S-methyltransferase-dependent methylation of DDTC to DDTC-Me (13), monooxygenase-dependent thionooxidation of DDTC-Me to DDTC-Me sulfone (7, 9), methyl ester sulfone; DETC-Me, S-methyl N,N-diethyldithiocarbamate; DETC-Me sulfoxide, diethyldithiocarbamate methyl ester sulfoxide; CYP, cytochrome P450; DETC-Me sulfoxidation, diethyldithiocarbamate methyl ester sulfoxidation; DETC-Me methylation, S-methyl N,N-diethyldithiocarbamate sulfoxidation; K_M, apparent K_M.
glutathione and glutathione S-transferase-dependent desulfuration of DDTC-Me sulfoxide to DETC-Me sulfoxide (14), and finally cytochrome P450-dependent sulfoxidation of DETC-Me to DETC-Me sulfoxide (4, 5).

The thionooxidation of DDTC-Me and the sulfoxidation of DETC-Me have been shown to be catalyzed primarily by cytochrome P450 enzymes (4, 5, 7, 9). CYP2B1 is particularly effective in catalyzing the sulfoxidation of DETC-Me (5), although this enzyme is virtually absent in liver microsomes obtained from untreated rats (15), and CYP2B6, the corresponding human cytochrome P450 enzyme has been shown to be functionally expressed at only low levels in a very small percentage of individuals (16, 17).

The present study was conducted to examine further the effect of cytochrome P450 induction on DETC-Me sulfoxidation in rats, and to identify the human cytochrome P450 enzymes responsible for DETC-Me sulfoxidation. Techniques used included induction of rat cytochrome P450 enzymes, use of cDNA-expressed human cytochrome P450 enzymes, correlation analysis of the sample-to-sample variation in human cytochrome P450 enzyme activities, and chemical and antibody inhibition. Our findings suggest that DETC-Me sulfoxidation is catalyzed by multiple cytochrome P450 human enzymes, including CYP2A6, CYP2E1, and CYP3A4/5.

Materials and Methods

DETC-Me sulfoxide, DETC-Me, and ethiolate sulfoxide were synthesized by established methods (3, 18, 19). Microsomes prepared from a cell line expressing a single cytochrome P450 enzyme were obtained from Gentest Corp. (Woburn, MA). These microsomes were prepared from the human lymphoblastoid cell line, AHH-1 (originally derived from RPMI 1788 cell line), that was transfected with cDNA encoding a human cytochrome P450 enzyme. The antibodies against CYP2E1 (20, 21) were a generous gift from Dr. Dennis R. Koop (Department of Pharmacology and Toxicology, Oregon Health Sciences University, Portland, OR). Sulphaphenazole was obtained from Ciba-Giegy Ltd. (Basel, Switzerland). Troleandomycin was obtained from Pfizer, Inc. (Brooklyn, NY). HPLC solvents were purchased from Fisher Scientific Co. (St. Louis, MO). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Human liver microsomes (Hepatocytes Test kit) were obtained from Human Biologics, Inc. (Phoenix, AZ), which also provided information on the cytochrome P450 enzyme activity in each sample. Rat liver microsomes from treated or untreated rats were also obtained from Human Biologics.

Antibodies against CYP3A1 and CYP1A1. The antibodies against rat CYP1A1 and CYP3A1 enzymes were raised in male New Zealand White rabbits as described by Thomas et al. (15, 22). The purification and immunosorbent absorption of these antibodies was conducted by previously described methods (23, 24).

DETC-Me Sulfoxidation. Sulfoxidation of DETC-Me to DETC-Me sulfoxide was conducted in 1.5-ml incubation mixtures that contained potassium phosphate buffer (100 mM, pH 7.4), EDTA (1 mM), microsomes (0.067–0.67 mg/ml protein), NADP⁺ (0.67 mM), glucose 6-phosphate (6.67 mM), and glucose 6-phosphate dehydrogenase (0.67 units/ml) with or without other additions as described in Results. The reactions were initiated by the addition of DETC-Me in 15-μl acetonitrile (final concentration, 100 μM). Incubations were conducted for 30 min in a shaking metabolic incubator. The reaction was terminated by the addition of CaCl₂ (final concentration, 20 mM). Incubations to which CaCl₂ was added immediately after the addition of the substrate served as time 0.

HPLC Analysis of DETC-Me Sulfoxidation. After the reactions were terminated, the precipitated protein was removed by centrifugation. Ethiolate sulfoxide (15 μl of 10 mM stock in acetonitrile) was added to 1.5 ml of the supernatant fraction as an internal standard. The supernatant fraction was then loaded on a 1-ml Extract-Clean column (containing 100 mg of C₁₈ solid phase) that had been washed with 1 ml of methanol and equilibrated with 1 ml of 100 mM potassium phosphate buffer (pH 7.4). The columns were washed with 0.5 ml of distilled water and then eluted with 0.3 ml of acetonitrile. The samples were diluted with 0.3 ml of water and then analyzed by HPLC at room temperature. The HPLC system consisted of a Beckman/Altex C₁₈ column (250 × 4.6 mm, 5 μm particle size), Shimadzu LC-10A system with a UV detector (215 nm). The following gradient program was used: a 25% mixture (v/v) of acetonitrile in water for the first 4 min, after which the acetonitrile content was linearly increased to 50% v/v over a period of 1 min, and maintained for 10 min. The flow rate was 1 ml/min. Data acquisition was conducted with a Shimadzu EZCHROM (version 2.1) program. A multiple-point calibration curve was made from known amounts of standards dissolved in 100 mM of potassium phosphate buffer (pH 7.4) that were carried through the extraction procedure. Calibration curves were linear in the concentration range studied. Retention times of DETC-Me sulfoxide, ethiolate sulfoxide, and DETC-Me were 3.2, 3.8, and 10.2 min, respectively. The extraction efficiencies based on measurements with spiked samples were 74%, 72%, and 100% for DETC-Me sulfoxide, ethiolate sulfoxide, and DETC-Me, respectively. The detector response was linear in the concentration range studied.

Results

DETC-Me Suloxidation by Liver Microsomes from Rats Treated with Various Cytochrome P450 Enzyme Inducers. The effect of treating rats with various cytochrome P450 inducers on liver microsomal DETC-Me suloxidation is shown in Table 1. Treatment of rats with the CYP2B inducers phenobarbital and 2,4,5,2',4,5'-hexachlorobiphenyl caused a 9- and 7.5-fold increase in DETC-Me suloxidation, respectively; whereas, treatment with the CYP1A inducer, 3-methylcholanthrene, resulted in a 2-fold increase. These data suggested that DETC-Me suloxidation can be catalyzed by both CYP2B and CYP1A enzymes. When rats were treated with the CYP3A inducers, pregnenolone-16α-carbonitrile and dexamethasone, or the CYP2E1 inducer isoniazid, an increase in liver microsomal DETC-Me suloxidation activity was observed. However, this increase was not statistically significant. Conversely, liver microsomes from hypophysectomized male rats and untreated female rats catalyzed the sulfoxidation of DETC-Me at a slower rate than those from untreated male rats. This difference was also not statistically significant.

Hypophysectomy of male rats suppresses the expression of
Effect of cytochrome P450 enzyme inducers on DETC-Me sulfoxidation by liver microsomes from male rats

<table>
<thead>
<tr>
<th>Inducing Agent</th>
<th>DETC-Me Sulfoxidation (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>24.8 ± 1.8*</td>
</tr>
<tr>
<td>2,4,5',4',5'-Hexachlorobiphenyl</td>
<td>20.2 ± 1.8*</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>5.6 ± 0.4*</td>
</tr>
<tr>
<td>Pregnenolone-16α-carbonitrile</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>Hypophysectomized males</td>
<td>2.0 ± 0.08</td>
</tr>
<tr>
<td>Female control</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

Rats were treated with the respective enzyme inducers. The induced microsomes were obtained from Human Biologics. DETC-Me (0.1 mM) was incubated with 0.1 mg liver microsomal protein and an NADPH-generating system for 30 min at 37°C. DETC-Me sulfoxidation was determined by HPLC as described in Materials and Methods. Both male and female controls reflect microsomes from corn oil-treated rats. Female controls are given for comparison. Data represent mean ± SE of 4 determinations.

Values in parentheses indicate the fold increase compared with controls when this increase was statistically significant.

Effect of cytochrome P450 enzyme inducers on DETC-Me sulfoxidation by liver microsomes from female rats

<table>
<thead>
<tr>
<th>Inducing Agent</th>
<th>DETC-Me Sulfoxidation (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Troleandomycin + K3Fe(CN)6</td>
<td>7.8 ± 1.0*</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>4.0 ± 0.7</td>
</tr>
</tbody>
</table>

Rats were treated with the respective enzyme inducers. Induced microsomes were obtained from Human Biologics. DETC-Me (0.1 mM) was incubated with 0.1 mg liver microsomal protein and an NADPH-generating system for 30 min at 37°C. DETC-Me sulfoxidation was determined by HPLC as described in Materials and Methods. Controls reflect microsomes obtained from corn oil-treated rats. Data represent mean ± SE of 4 determinations.

Values in parentheses indicate the fold increase compared with controls when this increase was statistically significant.

CYP3A2 and CYP2C11, enzymes which are male specific in adult rats. Although, not statistically significant, there was a tendency for the CYP3A inducers to increase DETC-Me sulfoxidation. This finding, combined with the observation that DETC-Me sulfoxidation was slower in hypophysectomized male rats and in untreated female rats, suggested a possible role for CYP3A in the sulfoxidation of DETC-Me. Therefore, the effect of treating female rats with the CYP3A inducers, troleandomycin and dexamethasone, on DETC-Me sulfoxidation was examined (table 2). Troleandomycin not only induces CYP3A enzymes, but is also metabolized by them to a compound that forms a stable complex that inhibits CYP3A activity. This complex can be dissociated by K3Fe(CN)6, which restores CYP3A enzyme activity (25). DETC-Me sulfoxidation was unaltered in microsomes from troleandomycin-treated rats, but was increased up to 6-fold upon treatment of these microsomes with K3Fe(CN)6 (table 2). In contrast to male rats (table 1), treatment of female rats with dexamethasone caused a 3-fold increase in DETC-Me sulfoxidation (table 2). Although induction of CYP3A enzymes in male rats had no statistically significant effect on DETC-Me sulfoxidation, data from the studies using female rat liver microsomes provided strong evidence for the involvement of CYP3A enzymes in DETC-Me sulfoxidation. Therefore, studies using liver microsomes from both male and female rats suggested that CYP1A1/2, CYP2B1/2, and CYP3A1/2 enzymes can catalyze DETC-Me sulfoxidation.

Determinations of KM(app) and Vmax for DETC-Me Sulfoxidation. A pool of five human liver microsomes was used to determine the Michaelis constants for DETC-Me sulfoxidation. The Lineweaver-Burk plot (fig. 2) suggested that human liver microsomes contain at least one high-affinity [KM(app) ~ 23 μM] and one low-affinity enzyme [KM(app) ~ 435 μM] that catalyze the sulfoxidation of DETC-Me. Because DETC-Me is found at a very low concentration in human plasma after disulfiram administration (26), only the high-affinity enzyme(s) may be clinically relevant. A concentration of 100 μM of DETC-Me was chosen to study the contribution of different cytochrome P450 enzymes. It is recognized that, at this concentration, the low-affinity enzyme will have a significant contribution toward DETC-Me sulfoxidation. Lower concentrations of DETC-Me were not studied because of low sensitivity with the HPLC assay that would have made data interpretation difficult.

DETC-ME Sulfoxidation by cDNA-Expressed Cytochrome P450 Enzymes. The rate of DETC-Me sulfoxidation by cDNA-expressed human enzymes is shown in table 3. All of the human cytochrome P450 enzymes examined catalyzed the sulfoxidation of DETC-Me. In the case of CYP3A4, the sulfoxidation of DETC-Me was barely detectable, unless the enzyme was expressed with additional NADPH-cytochrome P450 reductase. These data raise the possibility that the basal amount of NADPH-cytochrome P450 reductase in lymphoblastoid cells may be insufficient to support maximal rates of DETC-Me sulfoxidation by other cDNA-expressed cytochrome P450 enzymes. Therefore, this complicates the interpretation of the data shown in table 3.

Shimada et al. (17) recently estimated the average amount of each cytochrome P450 enzyme present in human liver microsomes. These averages, combined with the data obtained from the cDNA-expressed enzymes, were used to estimate the contribution of each cytochrome P450 enzyme to DETC-Me sulfoxidation by human liver microsomes (table 3). The estimated contribution of cytochrome P450 enzymes that catalyze DETC-Me sulfoxidation by human liver microsomes followed the rank order: CYP3A4 > CYP2C9 > CYP1A2 > CYP2E1 > CYP2A6. All other enzymes seem to contribute negligibly toward DETC-Me sulfoxidation by human liver microsomes. However, this rank order may be distorted by the fact that the ratio of NADPH-cytochrome P450 reductase to cytochrome P450 varied among the cDNA-expressed enzymes. It is particularly noteworthy that CYP3A4 ranked first or last, depending on whether additional NADPH-cytochrome P450 reductase was expressed in the lymphoblastoid cells.

Correlation Analysis of the Sample-to-Sample Variation in Human Liver Microsomal Cytochrome P450 Activities with DETC-Me Sulfoxidation. DETC-Me sulfoxidation was studied in 13 samples of human liver microsomes obtained from Human Biologics.
A pool of five human liver microsomes (0.1 mg) was incubated with various concentrations of DETC-Me sulfoxide formed was determined by HPLC as described in Materials and Methods. DETC-Me sulfoxidation at concentrations of 10, 35, and 50 μM were used to determine the kinetic constants for the high-affinity enzymes. Using these kinetic constants, the contribution of the high-affinity enzyme was subtracted from the total DETC-Me sulfoxidation and the contribution of the low-affinity enzyme estimated.

The rate of DETC-Me sulfoxidation in these samples varied from 0.3 to 5.4 nmol/min/mg protein. As shown in fig. 3, the sample-to-sample variation in DETC-Me sulfoxidation correlated well with the sample-to-sample variation in coumarin 7-hydroxylation (r = 0.88), testosterone 6β-hydroxylation (r = 0.90), and diazepam 3-hydroxylation (r = 0.94). Testosterone 6β-hydroxylation (27, 28) and diazepam 3-hydroxylation (29, 30) are catalyzed primarily by CYP3A4/5 enzymes, whereas coumarin 7-hydroxylation is catalyzed primarily by CYP2A6 in human liver microsomes (31, 32). These results suggest that CYP3A4/5 and possibly CYP2A6 contribute significantly to the sulfoxidation of DETC-Me by human liver microsomes.

Correlation Analysis in the Absence or Presence of Selective Inhibitors of Cytochrome P450 Enzymes. Correlation analysis can be misleading if more than one enzyme contributes to the same reaction or if the amount of one enzyme parallels that of another enzyme. Studies were therefore conducted to determine if correlation analysis could be improved by using inhibitors of various cytochrome P450 enzymes. These included coumarin (inhibitor of CYP2A6), sulfaphenazole (inhibitor of CYP2C enzymes), DDTC (an inhibitor of CYP2A6 and CYP2E1 enzymes), and troleandomycin (inhibitor of CYP3A enzymes). With the exception of DDTC, which inhibits several cytochrome P450 enzymes at high concentrations (33), all other inhibitors were studied at concentrations that produced maximum inhibition of DETC-Me sulfoxidation in a pool of five human liver microsomes (data not shown). DETC-Me sulfoxidation by nine

### Table 3

<table>
<thead>
<tr>
<th>Cytochrome P450 Enzyme</th>
<th>DETC-Me Sulfoxidation</th>
<th>Predicted Average Relative Contribution of Cytochrome P450 Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pmol/Min/Mg</td>
<td>Min⁻¹ Pmol/P450/Mg</td>
</tr>
<tr>
<td>Control</td>
<td>N.D.</td>
<td>—</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>12</td>
<td>0.6</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>60</td>
<td>1.6</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>120</td>
<td>2.0</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>67</td>
<td>1.2</td>
</tr>
<tr>
<td>CYP2C9 + Red²</td>
<td>14</td>
<td>2.0</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>27</td>
<td>0.1</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>10</td>
<td>1.2</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>N.D.</td>
<td>—</td>
</tr>
<tr>
<td>CYP3A4 + Red²</td>
<td>220</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Microsomes (0.2 mg) prepared from control cells or cells transfected with cDNA encoding various cytochrome P450 enzymes were incubated with DETC-Me (0.1 mM) and an NADPH generating system for 60 min at 37°C. All incubations were conducted in duplicate. The amount of DETC-Me sulfoxide formed was determined by HPLC as described in Materials and Methods. ND, not detected.

The contribution of each enzyme was calculated based on the sum of the predicted rate of formation of DETC-Me sulfoxidation from all the cDNA-expressed enzymes studied.

CYP1A1 is not known to be expressed in human liver.

These microsomes were obtained from cell lines transfected with the cDNA of both the cytochrome P450 enzyme and the NADPH-cytochrome P450 reductase.

This includes the entire CYP2C family, whereas only CYP2C9 was used to study DETC-Me sulfoxidation.

samples of human liver microsomes was examined in the absence or the presence of inhibitor. DETC-Me sulfoxidation activity in the absence of inhibitor, the activity remaining, and the activity inhibited in the presence of inhibitor were correlated with the activities of various cytochrome P450 enzymes. The effect of coumarin on DETC-Me sulfoxidation is shown in fig. 4. Coumarin inhibited DETC-Me sulfoxidation by 30–60%. The rate of DETC-Me sulfoxidation that could be inhibited by coumarin correlated better with CYP2A6 (r = 0.76) than did the rate of DETC-Me sulfoxidation in the absence of coumarin (0.67). Conversely, the correlation of the residual activity was poorer. These results suggest that CYP2A6 does contribute significantly to the sulfoxidation of DETC-Me by human liver microsomes.

In the presence of coumarin, the correlation between the remaining DETC-Me sulfoxidation activity and testosterone 6β-hydroxylation (CYP3A activity) improved from 0.76 to 0.94; the correlation with chlorzoxazone 6-hydroxylation (CYP2E1) improved from 0.07 to 0.19.

![Lineweaver-Burk plot for DETC-Me sulfoxidation by human liver microsomes](image-url)
DETC-Me sulfoxidation was determined in 13 human microsome samples. Human liver microsomes (0.1 mg) were incubated with DETC-Me (0.1 mM; added in 15 μl acetonitrile) in the presence of an NADPH generating system for 30 min at 37°C. All incubations were conducted in duplicate. The amount of DETC-Me sulfoxide formed was determined by HPLC as described in Materials and Methods. DETC-Me sulfoxidation is expressed as nmol DETC-Me sulfoxide formed/min/mg protein. The cytochrome P450 enzyme activities are shown on the ordinate scale and are expressed as percentage of the maximum activity observed in 13 human liver microsomes. The maximum activities for various cytochrome P450 enzymes were: ethoxyresorufin O-deethylation, 95 pmol/min/mg; coumarin 7-hydroxylation, 3400 pmol/min/mg; tolbutamide methyl hydroxylation, 280 pmol/min/mg; S-mephenytoin 4'-hydroxylation, 200 pmol/min/mg; dextromethorphan O-demethylation, 300 pmol/min/mg; chlorzoxazone 6-hydroxylation, 6900 pmol/min/mg; testosterone 6α-hydroxylation, 1590 pmol/min/mg; diazepam 3-hydroxylation, 2800 pmol/min/mg; lauric acid 12-hydroxylation, 1450 pmol/min/mg. 

r, Pearson’s product moment correlation coefficient. Correlation was statistically significant (p < 0.05) when r ≥ 0.65.

0.44, and the correlation with tolbutamide methylhydroxylation (CYP2C9) improved from 0.44 to 0.69 (fig. 4). These results suggest that CYP2A6, CYP3A4, and possibly CYP2C9 and CYP2E1 may contribute to DETC-Me sulfoxidation by human liver microsomes.

DDTC was first used as an inhibitor of CYP2E1 (34), but subsequent studies have shown it inhibits several human enzymes, including CYP2A6, CYP2B6, CYP2C8, and CYP3A4/5 (33). DDTC caused a 20–50% inhibition of the DETC-Me sulfoxidation by human liver microsomes (fig. 5). The DDTC-inhibitable rate of DETC-Me sulfoxidation correlated well with CYP2C9 (r = 0.83) and CYP2E1 (r = 0.50) activity. Therefore, DDTC, like coumarin, improved the correlation between DETC-Me sulfoxidation (i.e. the inhibited activity) and CYP2C9 and CYP2E1 activity. When the residual activity is considered, inhibition by DDTC did not improve the correlation between DETC-Me sulfoxidation and individual cytochrome P450 enzyme activities. This probably reflects the fact that DDTC inhibits several cytochrome P450 enzymes in human liver microsomes.

The CYP2C9 inhibitor, sulfaphenazole (35), caused little or no inhibition of DETC-Me sulfoxidation by human liver microsomes, regardless of the CYP2C9 content (as determined by tolbutamide methyl-hydroxylation) (fig. 6). This suggested that, contrary to the results shown in figs. 4 and 5, CYP2C9 does not contribute significantly to DETC-Me sulfoxidation by human liver microsomes.

The results obtained from use of inducers in rats (tables 1 and 2), use of cDNA-expressed enzymes (table 3), and correlation analysis (fig. 3) implicated CYP3A enzymes in DETC-Me sulfoxidation.
These data implicate CYP3A4/5, CYP2A6, and CYP1A2 enzymes in improved correlation with CYP1A2 and CYP2A6 activity enzyme activities. Values in boldface indicate that data are significant (p < 0.05), as determined by the Pearson’s product moment correlation analysis. r, correlation coefficient. Note: correlation coefficients obtained are slightly lower than shown in fig. 3. This is because of the difference in sample size between the studies.

Therefore, sample-to-sample variation in DETC-Me sulfoxidation was studied in the presence of troleandomycin, an inhibitor of CYP3A enzymes (25). Troleandomycin caused a 0–45% inhibition of DETC-Me sulfoxidation by human liver microsomes. The inhibited activity exhibited a better correlation (r = 0.91) with CYP3A4 activity, compared with DETC-Me sulfoxidation activity in the absence of inhibitor (r = 0.77). Conversely, the remaining activity showed an improved correlation with CYP1A2 and CYP2A6 activity (fig. 7). These data implicate CYP3A4/5, CYP2A6, and CYP1A2 enzymes in the sulfoxidation of DETC-Me by human liver microsomes.

Chemical Inhibition of DETC-Me Sulfoxidation. DETC-Me sulfoxidation was shown to be inhibited by coumarin, DDTC, and troleandomycin (figs. 4, 5, and 7), but not by sulfaphenazole (fig. 6). Other cytochrome P450 inhibitors also were examined for their effect on DETC-Me sulfoxidation (fig. 8). Quinidine, an inhibitor of CYP2D6 (36), and furafylline, an inhibitor of CYP1A2 (37, 38) did not inhibit DETC-Me sulfoxidation at the concentrations examined (fig. 8). α-Naphthoflavone, an inhibitor of CYP1A enzymes and an activator of CYP3A enzymes (39, 40), increased the rate of DETC-Me sulfoxidation up to 148% of the control rate (fig. 8). Ketoconazole, a potent inhibitor of CYP3A enzymes, with a Ki value in the submicromolar range (41), was found to be a relatively poor inhibitor of DETC-Me sulfoxidation (IC50 = 9 μM) (fig. 8). Ketoconazole is a nonselective inhibitor of cytochrome P450 enzymes at higher concentrations (41) and, thus, DETC-Me sulfoxidation was inhibited up to 60% by 10 μM of ketoconazole. The results in fig. 8 suggest that CYP1A2 and CYP2D6 enzymes do not contribute significantly to the sulfoxidation of DETC-Me by human liver microsomes. Although CYP3A4 can catalyze DETC-Me sulfoxidation, the weak inhibition with ketoconazole and troleandomycin suggested that other enzymes, such as CYP2A6 and CYP2E1, also contribute to this reaction.

Inhibition of DETC-Me Sulfoxidation by Polyclonal Antibod-
with DDTC. CYP1A enzymes contribute negligibly to DETC-Me sulfoxidation was not inhibited by antibodies against CYP1A, suggesting p- were shown to inhibit >80% of ethoxyresorufin O-dealkylase, inhibition studies (fig. 8). Anti-CYP2E1 inhibited up to 30% of human microsomes, respectively (data not shown). DETC-Me sulfoxidation by human liver microsomes. These antibodies anti-CYP2E1, and anti-CYP3A1 antibodies on the formation of nitrophenol hydroxylase, and testosterone 6/3-hydroxylase activities in DETC-Me sulfoxide by human liver microsomes. These antibodies inhibition studies thus provided additional evidence that several cytochrome P450 enzymes contribute to DETC-Me sulfoxidation by human liver microsomes in the absence and presence of sulphanephazole (bottom).

Human liver microsomes (0.1 mg) were incubated with DETC-Me (0.1 mM; added in 15 μl acetonitrile) and an NADPH generating system for 30 min at 37°C in the absence or presence of 10 μM sulphanephazole. All incubations were conducted in duplicate. The amount of DETC-Me sulfoxide formed was determined by HPLC as described in Materials and Methods. DETC-Me sulfoxidase activity in each of the nine human microsomal samples was correlated with cytochrome P450 activity as indicated. In addition, the DETC-Me sulfoxidase activity remaining in the presence of sulphanephazole and the activity inhibited by sulphanephazole were also correlated with cytochrome P450 enzyme activities. Values in boldface indicate that data are significant (p < 0.05), as determined by the Pearson’s product moment correlation analysis. r, correlation coefficient. Note: correlation coefficients obtained are slightly lower than shown in fig. 3. This is because of the difference in sample size between the studies.

Studies were conducted to determine the effect of anti-CYP1A1, anti-CYP2E1, and anti-CYP3A1 antibodies on the formation of DETC-Me sulfoxide by human liver microsomes. These antibodies were shown to inhibit >80% of ethoxyresorufin O-dealkylase, p-nitrophenol hydroxylase, and testosterone 6β-hydroxylase activities in human microsomes, respectively (data not shown). DETC-Me sulfoxidation was not inhibited by antibodies against CYP1A, suggesting that CYP1A enzymes contribute negligibly to DETC-Me sulfoxidation (fig. 9), a conclusion supported by the results from the chemical inhibition studies (fig. 8). Anti-CYP2E1 inhibited up to 30% of DETC-Me sulfoxidation activity in human microsomes (fig. 9). This is in agreement with the results from the chemical inhibition studies with DDTC.

DETC-Me sulfoxidation was inhibited only up to 40% by anti-CYP3A1 antibody. Even though this antibody inhibits testosterone 6β-hydroxylation completely >95% (data not shown), the possibility existed that the presence of the substrate DETC-Me may interfere with the binding of anti-CYP3A1 to CYP3A4/5. Therefore, anti-CYP3A1 was tested for its ability to inhibit DETC-Me sulfoxidation by cDNA-expressed CYP3A4. The results in fig. 9 show that anti-CYP3A1 completely inhibited the sulfoxidation of DETC-Me by cDNA-expressed CYP3A4, suggesting that DETC-Me does not interfere with the binding of anti-CYP3A1 to CYP3A4/5. The antibody-inhibition experiments thus provided additional evidence that several cytochrome P450 enzymes contribute to DETC-Me sulfoxidation by human liver microsomes.

**Discussion**

DETC-Me sulfoxidation has been proposed to be the terminal step in the bioactivation of disulfiram (3), a reaction that is catalyzed by
cytochrome P450 enzymes. However, this was not apparent when human liver microsomes were used. CYP1A2 did not seem to contribute to DETC-Me sulfoxidation, because this reaction could not be inhibited by sulfaphenazole, a known inhibitor of CYP2C9 (fig. 6). Similarly, CYP2C19 is not a major contributor, based on the poor correlation between S-mephentoin 4'-hydroxylation and DETC-Me sulfoxidation (fig. 3). In human liver microsomes, CYP2D6 also does not seem to contribute to DETC-Me sulfoxidation, because this reaction did not correlate with dextromethorphan O-demethylation (fig. 3) and was not inhibited by quinidine (fig. 8). A limited contribution of CYP2E1 was suggested as a result of the partial inhibition of DETC-Me sulfoxidation by DDTC and anti-CYP2E1 (fig. 5 and 9). Also, there was a modest improvement in the correlation between chlorzoxazone 6-hydroxylation (CYP2E1 activity) with residual DETC-Me sulfoxidation activity in the presence of coumarin and the DDTC-inhibitable rate of DETC-Me sulfoxidation (fig. 5). Several lines of evidence suggested that CYP3A enzymes contribute to DETC-Me sulfoxidation. These include: 1) a high correlation between DETC-Me sulfoxidation and testosterone 6β-hydroxylation (fig. 3); 2) an improvement in the correlation when the troleandomycin-inhibitable rate of DETC-Me sulfoxidation was compared with testosterone 6β-hydroxylation (fig. 7); 3) an improvement in the correlation when testosterone 6β-hydroxylation was compared with the residual activity of DETC-Me sulfoxidation in the presence of coumarin (fig. 4); 4) increased DETC-Me sulfoxidation in the presence of α-naphthoflavone, an activator of CYP3A enzymes (fig. 8); and 5) partial inhibition of DETC-Me sulfoxidation by antibodies against CYP3A enzymes (fig. 9). Collectively, these data suggest that CYP2A6, CYP2E1, and CYP3A4 enzymes all contribute substantially to the sulfoxidation of DETC-Me by human liver microsomes.

The finding that these cytochrome P450 enzymes contribute to the formation of DETC-Me sulfoxide, the proposed active metabolite of disulfiram, has important clinical implications. Although disulfiram has been used for the treatment of alcohol abuse for >40 years, the
daily dose generally required to produce a disulfiram-ethanol reaction is uncertain. For example, in a study by Brewer (42), only half of the subjects challenged with ethanol exhibited a disulfiram-ethanol reaction on a daily dose of 200–300 mg of disulfiram. Based on the complexity of bioactivation of disulfiram (fig. 1), we propose that the variable efficacy of disulfiram is caused by its faulty bioactivation, and, in humans, the amount of DETC-Me sulfoxide formed may be compromised, not only because of a deficiency in cytochrome P450 by DDTC. This implies that disulfiram may inhibit its own bioactivation. Furthermore, disulfiram is used in alcoholics undergoing this type of treatment (42, 44). The clinical use of disulfiram antifungals are potent inhibitors of CYP3A enzymes. These may also impair DETC-Me sulfoxidation, a reaction that is partially catalyzed by CYP3A. These studies now provide one possible explanation as to why disulfiram has a variable response pattern in many alcoholics and immunosuppressants are metabolized by similar cytochromes. Therefore, it is possible that DETC-Me sulfoxidation in humans may be compromised, not only because of a deficiency in cytochrome P450 enzymatic activity, but also because of the inhibition of cytochrome P450 by DDTC. This implies that disulfiram may inhibit its own bioactivation. Furthermore, disulfiram is used in alcoholics who are predisposed to liver disease, and therefore may have reduced levels of CYP1A2, CYP2E1, and CYP3A enzymes (43). In addition, several drugs presently used clinically (e.g. calcium channel blockers, antiarrhythmics, tranquillizers, antipsychotics, antihistamines, and immunosuppressants) are metabolized by similar cytochrome P450 enzymes. Thus, potential exists for competitive inhibition of DETC-Me sulfoxidation when these drugs are administered concomitantly with disulfiram. Also, some macrolide antibiotics and antifungals are potent inhibitors of CYP3A enzymes. These may also impair DETC-Me sulfoxidation, a reaction that is partially catalyzed by CYP3A. These studies now provide one possible explanation as to why disulfiram has a variable response pattern in many alcoholics undergoing this type of treatment (42, 44). The clinical use of DETC-Me sulfoxide directly should therefore eliminate much of the variability observed with disulfiram.

Acknowledgments. We are grateful to Dr. Dennis R. Koop for providing us with anti-CYP2E1 antibodies.

References


