3-Methylindole (3 MI) is a potent pneumotoxicant, found in tobacco smoke and intestinal or ruminant contents (Hoffman and Rathkamp, 1970; Carlson and Breeze, 1983; Yost, 1997). It requires bioactivation by cytochrome P450 (P450) enzymes before it elicits any toxic effects (Yost, 1989, 1997). The selective expression of certain cytochrome P450 enzymes in pulmonary tissue is a likely mechanism for the organ-selective toxicity (Gram, 1997) because metabolic bioactivation by P450 enzymes forms toxic electrophilic metabolites (Skordos et al., 1998a). Incubation of 3 MI with various vaccinia virus-expressed cytochrome P450s demonstrated (Thornton-Manning et al., 1996) that human P450s appeared to metabolize 3 MI to several bioactivation products, while other P450s would catalyze only the oxygenation of 3 MI. Therefore, the kinetics of product formation by the CYP2F1 and CYP2F3 enzymes were compared with other cytochrome P450 enzymes. The enzymes tested were CYP1A1, CYP1A2, CYP1B1, and CYP2E1. The CYP1A1 and CYP1A2 enzymes produced all three 3 MI metabolites: the dehydrogenation product, 3-methylenependolene ($V_{\text{max}}/K_m = 4$ and 22, respectively); the hydroxylation product, indole-3-carbinol ($V_{\text{max}}/K_m = 42$ and 100, respectively); and the epoxidation product, 3-methyloxindole ($V_{\text{max}}/K_m = 4$ and 72, respectively). These CYP1A enzymes catalyzed oxygenation of 3 MI at much faster rates than dehydrogenation. CYP1B1 produced indole-3-carbinol ($V_{\text{max}}/K_m = 85$) and 3-methyloxindole ($V_{\text{max}}/K_m = 7$), and CYP2E1 only produced 3-methyloxindole ($V_{\text{max}}/K_m = 98$), but neither enzyme catalyzed the formation of the dehydrogenated product. Six additional P450 enzymes that were tested formed none of the dehydrogenated product. The ability of the various CYP1 family enzymes to catalyze the formation of all three major 3 MI metabolites, along with the specific oxygenation by CYP2E1, illustrates that dehydrogenation of 3 MI is not a substrate-directed process, but that the members of the CYP2F family possess unique active sites that specifically catalyze only the dehydrogenation mechanism.

Examples of dehydrogenation of other substrates by P450 enzymes has generally been limited to aromatic compounds such as acetaminophen (Dahlin et al., 1984), butylated hydroxytoluene (Bolton and Thompson, 1991), and tamoxifen (Fan et al., 2000). However, the efficient dehydrogenation of “unactivated” alkanes has been demonstrated for chemicals such as ethyl carbamate by CYP2E1 (Lee et al., 1998); lauric acid by CYP2E1, CYP4A5/7, and CYP4B1 (Guan et al., 1998); and valproic acid (Rettie et al., 1995) by CYP4B1. Dehydrogenation of an azaindole-containing drug by rats, monkeys, and humans (Zhang et al., 2000), and an indole-containing drug, zafirlukast (Kassahun et al., 2000), by CYP3A enzymes, has also been recently documented. These examples validate the importance of the current studies that are designed to evaluate the catalytic preference of human P450 enzymes for the dehydrogenation versus oxygenation of a prototypical indole, 3 MI.

The objective of the current study was to determine whether the selective dehydrogenation of 3 MI is primarily a function of the substrate, or whether ring oxidation (3-methyloxindole) or methyl oxidation (indole-3-carbinol) can be the preferred route of metabolism by certain P450 enzymes. To that end, four P450s from outside the 2F subfamily were selected for metabolic/kinetic study using 3 MI as the substrate. These enzymes were chosen because they are expressed in human lung tissues and have been shown to bioactivate a variety of lung toxicants (Macé et al., 1998; Pelkonen and Raunio, 1997; Yost, 1997). Six additional P450 enzymes were evaluated at single saturating substrate concentrations of 3 MI to determine the
Materials and Methods

**Enzymes and Chemicals.** CYP1A1, CYP1A2, CYP1B1, and CYP2E1 containing human lymphoblastoid microsomes were a generous gift provided by Dr. Charles Crespi of GENTEST Corporation (Woburn, MA). The additional recombinant enzymes (CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP3A4, and CYP3A5) were purchased from GENTEST. 3-Methylindole, NADPH, and NAC were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade acetonitrile was purchased from a local supplier.

**Microsomal Incubations.** The microsomes were prepared by standard centrifugal methods. All incubations were done in duplicate incubations, and the results are expressed as an average of the duplicates. The reaction mixtures for 1A1, 1A2, 1B1, and 2E1 consisted of 250 pmol of P450, sodium phosphate buffer (0.05 M, pH 7.4), 2 mM NADPH, 4 mM NAC, and varying amounts of 3 MI. Incubations of the CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 enzymes contained 100 pmol of each enzyme, sodium phosphate buffer (0.05 M, pH 7.4), 2 mM NADPH, 4 mM NAC, and a single saturating concentration (200 μM) of 3 MI. The final volume of all incubations was 1 ml. Once mixed, the sample was incubated at 37°C for 10 min, which was within the linear portion of the product formation versus time curve for all enzymes. The reaction was stopped by addition of an equal volume (1 ml) of ice-cold acetonitrile. The sample was then centrifuged at 3000g for 20 min. The sample supernatant was dried to a volume of 200 μl, and then 20 μl of a 1 mM solution of 3-phenyloxindole was added to the mixture to serve as an internal standard.

**HPLC Analysis.** An aliquot of 100 μl was injected into the HPLC system. HPLC was performed on a Beckman Coulter, Inc. (Fullerton, CA) system composed of dual 114 M solvent pumps, a 412A controller, and a 210A injector connected to a Hewlett Packard (Palo Alto, CA) 1040A diode-array UV detector. The 3 MI metabolites were separated on a 5-μm reverse-phase Phenomenex (Torrance, CA) Ultremex C18 column (250 × 4.6 mm) using a gradient solvent system that began at 10% acetonitrile and 90% 0.1 M ammonium acetate buffer, pH 6.0, and then changed to 50% acetonitrile over 5 min, 55% acetonitrile over another 5 min, and a final change to 95% acetonitrile over 5 min using a flow rate of 1 ml/min. The chromatograms and spectra were monitored by ultraviolet absorption at 254 and 280 nm. The data were analyzed on a model 9000 Series 300 Hewlett Packard computer using HP79995A operating software. A representative chromatogram from the incubation of 3 MI with CYP1A1 is shown in Fig. 2. The retention times were as follows: 3 MINAC, 8.8 min; indole-3-carbinol, 10.6 min; 3-methyloxindole, 11.9 min; and 3-phenyloxindole (internal standard), 17 min. All other peaks in the chromatogram were present in control incubations that did not include NADPH. Peak ratios of authentic standards to the internal standard (3-phenyloxindole) were used to generate a standard curve, and this curve was used to determine the amounts of each of the three metabolites.

**Results and Discussion.** A typical HPLC chromatogram is shown in Fig. 2 to illustrate separation of the products. Apparent kinetic constants of 3 MI for the CYP2F1 (Lanza et al., 1999), CYP2F3 (Wang et al., 1998), and goat lung microsomes (Skiles and Yost, 1996) have been published previously. The CYP1A1 and CYP1A2 enzymes produced all three of the major 3 MI metabolites: the dehydrogenation product, 3-methylenedolene (trapped as its NAC adduct); the hydroxylation product, indole-3-carbinol; and the epoxidation product, 3-methyloxindole (Table 1). However, the apparent enzyme efficiencies (Vmax/Km) for the CYP1A enzymes showed that these enzymes catalyzed oxygen-
Incubations of 3 MI with recombinant P450 enzymes or microsomes were conducted as described under Materials and Methods. Apparent kinetic constants were determined using a second-order polynomial curve fitting analysis of substrate concentration versus product formation rate using the Michaelis-Menten equation with KaleidaGraph software (Synergy Software, Reading, PA). The apparent $K_m$ values are expressed as millimolar concentration, and apparent $V_{max}$ values are expressed as nanomoles of product per nanomoles of P450 per minute.

### Table 1

**Apparent kinetic constants for dehydrogenation versus oxygenation of 3 MI**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>3MINAC (Dehydrogenated Product)</th>
<th>Indole-3-carbinol</th>
<th>3-Methyloxindole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$V_{max}$ (nM/min/M)</td>
<td>$V_{max}/K_m$</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>0.54</td>
<td>2.2</td>
<td>4</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>0.26</td>
<td>5.7</td>
<td>22</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2F1</td>
<td>0.02</td>
<td>1.3</td>
<td>65</td>
</tr>
<tr>
<td>CYP2F3</td>
<td>0.34</td>
<td>0.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Goat lung microsomes$^c$</td>
<td>0.06</td>
<td>8.7</td>
<td>145</td>
</tr>
</tbody>
</table>

N.D., not detected.

$^a$ Data from Lanza et al., 1999.

$^b$ Data from Wang et al., 1998.

$^c$ Data from Skiles and Yost, 1996.

* CYP1B1 and CYP2E1 enzymes did not form any detectable 3-methylindole via the dehydrogenation pathway.

* CYP1B1 produced both oxygenated products, indole-3-carbinol and 3-methyloxindole. Previous studies with pulmonary microsomes (Skiles and Yost, 1996) demonstrated that both indole-3-carbinol and 3-methylindole are probably produced through a common radical intermediate after hydrogen abstraction from 3 MI. Thus, the specificity of the 1B1 enzyme to form the oxygen-rebound product, indole-3-carbinol, without forming any detectable dehydrogenation product, dramatically illustrated the concept that the active-site environment of 1B1 controls partitioning of the putative indolymethyl radical intermediate to oxygenation rather than dehydrogenation. This specificity for oxygenation was exactly the converse of the results for the 2F enzymes, demonstrating that the enzyme active sites of the 2F enzymes favor a second one-electron oxidation step from the indolymethyl radical, rather than an oxygen-rebound step. Dehydrogenation of other substrates has been documented for certain enzymes, such as CYP4B1 (Rettie et al., 1995; Guan et al., 1998) and CYP2E1 (Lee et al., 1998).

* CYP2E1 specifically produced only 3-methyloxindole, presumably through the 2,3-epoxide (Skordos et al., 1998b). Thus, this enzyme provides a different, catalytically distinct process of ring oxidation, without production of metabolites that involve methyl oxidation (to form either indole-3-carbinol, 3-methylindole, or both) that were produced by the 1A1, 1A2, 1B1, 2E1, 2F1, and 2F3 enzymes. Incubations with a single saturating substrate concentration of 3 MI with the other six P450 enzymes were included to determine whether any produced the dehydrogenated product; none did. The CYP2A6, CYP2C19, and CYP2D6 enzymes produced both indole-3-carbinol and 3-methyloxindole, whereas the CYP2B6, CYP3A4, and CYP3A5 enzymes formed only 3-methyloxindole like CYP2E1. Apparent rate constants were not determined for these additional P450 enzymes.

* These results confirmed the hypothesis that dehydrogenation of 3 MI is restricted to a relatively small number of P450 enzymes, and all of these enzymes are expressed selectively (CYP2F1 and CYP2F3) in lung tissues or are expressed in lung (Mace´ et al., 1998) in addition to other tissues (CYP1A1 and CYP1A2). The ability of the various cytochrome P450 enzymes to specifically form oxygenated 3 MI metabolites shows that dehydrogenation is not substrate driven. This fact would indicate that the CYP2F1 and CYP2F3 enzymes possess unique active sites that specifically direct oxidation to produce the dehydrogenated reactive intermediate. The unique active site environment of the 2F enzymes that specifically catalyze the dehydrogenation of 3 MI is the subject of ongoing studies in our laboratory.

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### References


