Cytochromes P450 with Bisallylic Hydroxylation Activity on Arachidonic and Linoleic Acids Studied with Human Recombinant Enzymes and with Human and Rat Liver Microsomes

JOHAN BYLUND, TINA KUNZ, KARIN VALMSEN and ERNST H. OLIW
Division of Biochemical Pharmacology, Department of Pharmaceutical Biosciences, Uppsala Biomedical Center, Uppsala University, Uppsala, Sweden
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ABSTRACT
Bisallylic carbons of polyunsaturated fatty acids can be hydroxylated in NADPH-dependent reactions in liver microsomes. Human recombinant cytochromes P450 and human and rat liver microsomes were assayed for bisallylic hydroxylation activity. CYP1A2, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 converted [14C]linoleic acid to 14C-labeled 11-hydroxyoctadecadienoic acid (11-HODE), whereas [14C]arachidonic acid was oxygenated by CYP1A2 and CYP3A4 to 14C-labeled 13-hydroxyeicosatrienoic acid (13-HETE), 10-HETE and 7-HETE as determined by HPLC. Both substrates were also converted to many other metabolites. CYP2C9 appeared to form 12R-HETE and 13-HETE, whereas CYP2C8 formed 13-HETE, 11-HETE and 15-HETE as main monohydroxy metabolites. Fetal human liver microsomes metabolized linoleic acid to 11-HODE as a major hydroxy metabolite, whereas arachidonic acid appeared to be hydroxylated at C13, C20 and, to some extent, at C10, C19 and C7. Fetal liver microsomes mainly formed 13R-HETE, whereas adult human liver microsomes and CYP1A2 mainly formed 13S-HETE. 7,8-Benzoflavone (5 μM) and furafylline (20 μM), two inhibitors of CYP1A2, reduced the bisallylic hydroxylation activity of adult human liver microsomes. Treatment of rats with erythromycin or dexamethasone induced bisallylic hydroxylation of linoleic acid to 11-HODE in liver microsomes by 2- and 10-fold, respectively. The biosynthesis of 11-HODE by microsomes of dexamethasone-treated rats was inhibited by troleandomycin (ED50 = 1 μM) and by polyclonal antibodies against CYP3A1, suggesting that CYP3A1 could catalyze bisallylic hydroxylations in the dexamethasone-treated rat. We conclude from steric analysis of 13-HETE and the effects of CYP inhibitors on adult human liver microsomes that CYP1A2 might contribute to its bisallylic hydroxylation activity.

Liver and renal cortical microsomes and NADPH oxygenate polyunsaturated fatty acids to a large number of metabolites (Capdevila et al., 1995; Oliw, 1994; Oliw et al., 1996). Many of the oxidizing enzymes have been identified as members of the P450 superfamily of heme-thiolate enzymes. Hydroxylations of carbons near or at the ω end and epoxidations of the double bonds by CYPs have attracted the largest attention due to the biological activities of these metabolites of arachidonic acid (Capdevila et al., 1995; McGiff, 1991; Oliw, 1994; Oliw et al., 1996). The CYP enzymes are important for metabolism of other endogenous compounds like vitamin D, steroids, bile acids, prostaglandins and leukotrienes as well as xenobiotics (Dennis and Whitlock, 1995; Nelson et al., 1996). Some CYP enzymes catalyze more or less regiospecific oxygenations with fatty acids as substrates. The CYP4A subfamily catalyzes ω1 and ω2 hydroxylation of polyunsaturated fatty acids (Capdevila et al., 1995; Oliw, 1994), whereas CYP2E1 and other isozymes have been reported to catalyze ω2 and ω3 hydroxylations of arachidonic acid (Laethem et al., 1993; Rifkind et al., 1995). Some human and rodent epoxygenases have also been identified in the liver and kidney. For example, CYP1A2 and isoforms of CYP2B and CYP2C catalyze epoxygenation of arachidonic acid (Capdevila et al., 1995; Daikh et al., 1994; Oliw, 1994; Zeldin et al., 1995), but it seems likely that many other constitutive enzymes with epoxygenase activity remain to be identified both in liver and in extrahepatic organs.

Liver microsomes can catalyze two additional NADPH-dependent oxygenation reactions: bisallylic hydroxylation...
and hydroxylation with double bond migration (Oliw et al., 1993). Bisallylic hydroxylation in liver microsomes of phenobarbital-treated rats converts linoleic acid to 11-HODE and arachidonic acid to 7-HETE, 10-HETE and 13-HETE (Brash et al., 1995; Hörnsten et al., 1996; Oliw et al., 1993). These metabolites are acid labile and decompose to cis-trans conjugated dienols nonenzymatically (Hamberg et al., 1992). Hydroxylation with double bond migration, which also has been referred to as allylic hydroxylation (Capdevila et al., 1995), leads to enzymatic biosynthesis of cis-trans conjugated hydroxy fatty acids. The mechanism has been investigated in microsomes of phenobarbital-treated rats with linoleic acids stereospecifically deuterated at C11 as substrates (Oliw et al., 1993). Linoleic acid was converted to 9-HODE and 13-HODE with 80% to 82% R stereochemistry by initial hydrogen abstraction at C11 with subsequent double bond migration and oxidation at C9 or C13. The same system oxygenated arachidonic acid to 12-HETE (80% R) in moderate amounts. Human liver microsomes can also form 13-HETE and 12R-HETE (Oliw, 1993). The mechanism of biosynthesis of 12R-HETE by liver microsomes has not been determined, but it seems likely that it occurs by hydroxylation with double bond migration. An alternative route may be present in skin, in which 12R-HETE might be formed by other mechanisms (McGiff, 1991; Oliw, 1994). 12R-HETE is a partial agonist of the leukotriene B4 receptor and it has therefore attracted some biological attention (McGiff, 1991).

The CYP enzymes that catalyze bisallylic hydroxylation and hydroxylation with double bond migration have not been identified. Phenobarbital treatment of rats increased the bisallylic hydroxylation activity of liver microsomes by only a little. Treatment of rats with DEX, which mainly induces the CYP3A subfamily in the rat liver (Pereira and Lechner, 1995), increased the bisallylic hydroxylation activity of liver microsomes on linoleic, arachidonic and eicosapentaenoic acids (Hörnsten et al., 1996). Bisallylic hydroxylation activity could also be increased by treatment of rats with acetone plus starvation, with imidazole and with β-naphthoflavone, indicating that other enzymes than the CYP3A subfamily could have this enzyme activity in the rat.

The CYP enzymes with bisallylic hydroxylation activity can be conveniently identified with the help of recombinant enzymes. Bisallylic hydroxylation activity of human recombinant CYP has not been investigated, but a recent report addresses this question indirectly. Rifkind et al. (1995) found that CYP1A2, CYP2C8 and CYP2C9 converted arachidonic acid to relatively large amounts of cis-trans conjugated HETEs. In this study, it is likely that bisallylic hydroxy metabolites may have decomposed to cis-trans conjugated HETEs during the acidic extractive isolation. These investigators made the interesting observation that CYP2C9 metabolized arachidonic acid to 12-HETE, but the stereochemistry of 12-HETE was not examined.

The first objective of the present study was to investigate whether a series of recombinant human CYP enzymes could form bisallylic hydroxy metabolites of linoleic and arachidonic acids. Linoleic acid is a convenient substrate for this purpose, whereas arachidonic acid can be oxygenated to a much more complex mixture of metabolites. Biosynthesis of 12-HETE by CYP2C9 and its allelic variant R144C was also investigated. The second objective was to use the results from the screening of the CYP enzymes to study their contribution to the bisallylic hydroxylation activity of adult human liver microsomes. This was done by the aid of enzyme inhibitors and by chiral analysis of the main product, 13-HETE. Thus, we examined the effects of some enzyme-specific inhibitors on bisallylic hydroxylation activity of adult human liver microsomes and compared the chirality of 13-HETE formed by CYP and by liver microsomes. We also found that human fetal liver microsomes catalyzed bisallylic hydroxylation of fatty acids. Finally, the effects of gender, starvation and treatment with ERY and DEX on rat liver bisallylic hydroxylation activity were examined.

### Experimental Procedures

**Materials.** 18:2n-6 (99%), 20:4n-6 (99%), DEX, phenylmethylsulfonyl fluoride, testosteron, 7,8-benzoflavone, ERY and TAO were from Sigma Chemical (St. Louis, MO). [1-14C]18:2n-6, [1-14C]20:4n-6 (55 Ci/mol) and [4-14C]testosterone (56 Ci/mol) were from Amersham (Amersham, UK). The radiolabeled fatty acids were usually diluted with unlabeled fatty acids to a specific activity of 2.3 Ci/mol. 6β-Hydroxytestosteron was from Steraloids (Wilton, NH). Cartridge extraction (SepPak/C_{18}) were from Waters (Milford, MA). 13S-HODE and 15S-HETE were obtained through biosynthesis (Oliw et al., 1993). 9R,S-HETE, 11R,S-HETE, 12S-HETE and 20-R-HETE were from Cayman Chemical (Reading, UK). 12R-HETE methyl ester was a gift of Dr. S.-E. Dahlén (Karolinska Institutet, Stockholm, Sweden). 18R-HETE and 19R-HETE were obtained by biosynthesis with mycelia of Glaumennymycycin granimis (Oliw, 1989), whereas 17β-estradiol was a gift from Dr. J. R. Falek (University of Texas Southwestern Medical Center, Dallas, TX). Sulfaphenazole and furafylline were purchased from Ultrafine Chemicals (Manchester, UK). Microsomes of recombinant human P450 expressed in lymphoblastoid cells (CYP1A2, CYP2A6, CYP2B6, CYP2D6 and CYP2E1) and insect cells [CYP3A4, CYP2C8, CYP2C9 (with Arg144 or Cys144) and CYP2C19] were purchased from Gentest (Woburn, MA). Three samples of human livers were obtained from the liver bank of Huddinge Hospital (courtesy of Dr. J. Säwe and Dr. M.-L. Dahl): HL37 was from a 59-year-old woman who died from head trauma after treatment with mannitol, euvacillin and atropine; HL42 from a 31-year-old man who died after subarachnoidal bleeding after treatment with theophyllin and phenytoin; and HL46 was from an adult male with an unknown case history. Two samples of human fetal livers (before week 24 of gestation) were kindly provided by Dr. A. Rane (Akademiska Sjukhuset, Uppsala, Sweden). Polyvalent rabbit antibodies against CYP3A1 were purchased from XenoTech (Kansas City, KS) and were used for inhibition of CYP3A1 as recommended by the supplier.

**Incubation with microsomes of recombinant human P450 expressed in insect or lymphoblastoid cells.** From 25 to 50 pmol of CYP3A4, CYP2C8, CYP2C9 or CYP2C19 in 0.5 to 1 ml of incubation buffer (0.05 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol) were incubated with [3H]-labeled fatty acids (4–5 μM; 56 Ci/mol) and NADPH (1 mM) for 30 min at 37°C. P450 expressed in lymphoblastoid cells were incubated for 2 hr at 37°C (~30–65 pmol of CYP1A2 and CYP2A6, 45–90 pmol of CYP2E1, 65–130 pmol of CYP2D6 and 50–100 pmol of CYP2B6) with 15 to 20 μM of substrate. Microsomes of insect and lymphoblastoid cells without expressed P450 were incubated in the same way. Incubations were terminated by four volumes of ethanol. An internal standard (13-HODE or 15-HETE) was added to check for recovery on HPLC and used for estimation of biosynthesis of metabolites during the incubation time period and for comparison between enzymes (tables 1 and 2). The standards also verified the recovery times on HPLC.

**Incubation with human and rat liver microsomes.** Human liver microsomes were prepared by differential centrifugation as described and stored at −80°C until use (Oliw, 1990). Rat liver microsomes were prepared separately in the same way from each
animal. Fischer 344 rats (170–190 g; Mollegaard, Skensved, Denmark) were treated with DEX (150 mg/kg for 4 days; three males, one female), with ERY (734 mg/kg p.o. for 5 days, n = 3) or with solvent (1% Tween-20; three males, one female) as described previously (Daujat et al., 1991). Three male rats were also starved for 48 hr.

Microsomes were suspended in 1 to 2 ml of incubation buffer as described above to a protein concentration of about 1 mg/ml and incubated with 1.0 mM NADPH and the 14C-labeled fatty acids (usually 0.1 mM, 2.3 Ci/mmol) for 30 min at 37°C with constant shaking. Incubations were terminated as above. The effects of mechanism-based inhibitors (furafylline and TAO; both dissolved in methanol) were assessed after preincubation with the drug and NADPH for 15 min at 37°C. [14C]18:2 or [14C]20:4 was then added to the incubation, which was terminated after 30 min as described above. Sulfa-phenazole and 7,8-benzoflavone were added directly to the incubations without preincubations. Control incubations and incubations with drugs were performed in duplicate.

### Purification of metabolites

After extractive isolation without acidification on a cartridge with octadecasilane silica (SepPak/C18), the arachidonic acid metabolites were first separated by RP-HPLC into three groups of metabolites: triols and diols, HETEs and epoxides. HETEs typically eluted between 20 and 30 min. These fractions with HETEs were then pooled and analyzed by SP-HPLC. 13-HETE and 19-HETE have similar retention times on RP-HPLC (Brash et al., 1995), and in some experiments, unlabeled 19R-HETE (25–50 µg; monitored by UV at 207 nm) was added to localize the 13-HETE metabolite in the chromatogram. Unlabeled 12-HETE was added to incubations with CYP2C9 to facilitate identification of radiolabeled 12R-HETE by RP-HPLC, SP-HPLC and chiral HPLC. The metabolites of linoleic acid were extracted as above and separated by RP-HPLC. The 11-HODE peak was further analyzed by SP-HPLC and GC-MS in some experiments.

### HPLC

The equipment for HPLC has been described (Hörnsten et al., 1996). In short, the columns contained octadecasilane silica (5-

### TABLE 1

<table>
<thead>
<tr>
<th>CYP</th>
<th>Incubation time</th>
<th>Rate</th>
<th>Percent of total metabolites</th>
<th>Epoxides/diols</th>
<th>11-HODE</th>
<th>ω2</th>
<th>ω1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>120 min</td>
<td>0.06</td>
<td>41%</td>
<td>17%</td>
<td>22%</td>
<td>14%</td>
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<tr>
<td>CYP2C8</td>
<td>30 min</td>
<td>0.3</td>
<td>50%</td>
<td>8%</td>
<td>5%</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>30 min</td>
<td>0.6</td>
<td>62%</td>
<td>5%</td>
<td>2%</td>
<td>18%</td>
<td></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>30 min</td>
<td>0.2</td>
<td>54%</td>
<td>4%</td>
<td>23%</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>30 min</td>
<td>0.4</td>
<td>37%</td>
<td>28%</td>
<td>6%</td>
<td>13%</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Liver microsomes</th>
<th>Rate</th>
<th>Epoxides/diols</th>
<th>HETEs</th>
<th>12-HETE</th>
<th>13-HETE</th>
<th>ω2</th>
<th>ω1</th>
<th>Bis-HETEs and other HETEs&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL37</td>
<td>0.18</td>
<td>35</td>
<td>8</td>
<td>7</td>
<td>32</td>
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<td></td>
<td>19</td>
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<tr>
<td>HL42</td>
<td>0.19</td>
<td>31</td>
<td>6</td>
<td>6</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL46</td>
<td>0.19</td>
<td>30</td>
<td>6</td>
<td>6</td>
<td>37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL2</td>
<td>0.11</td>
<td>33</td>
<td>19</td>
<td>6</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<sup>a</sup> CYP1A2 was expressed in lymphoblastoid cells, whereas the others were expressed in insect cells together with NADPH-P450 reductase. The CYP3A4 was also expressed with cytochrome b<sub>5</sub>. In all experiment, unidentified polar metabolites were also formed (<20% of total metabolites), and some of these were also present in control cells without expressed CYP. Liver microsomes were incubated for 30 min.

### TABLE 2

<table>
<thead>
<tr>
<th>Liver microsomes</th>
<th>Rate</th>
<th>Epoxides/diols</th>
<th>HETEs</th>
<th>13-HETE</th>
<th>12-HETE</th>
<th>ω2</th>
<th>ω1</th>
<th>Bis-HETEs and other HETEs&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL37</td>
<td>0.35</td>
<td>37</td>
<td>41</td>
<td>8</td>
<td>14</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL42</td>
<td>0.31</td>
<td>31</td>
<td>32</td>
<td>3</td>
<td>13</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL46</td>
<td>0.36</td>
<td>28</td>
<td>27</td>
<td>4</td>
<td>8</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL1</td>
<td>N.D.</td>
<td>30</td>
<td>52</td>
<td>2</td>
<td>18</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL2</td>
<td>0.23</td>
<td>27</td>
<td>41</td>
<td>2</td>
<td>10</td>
<td>29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> bis-HETEs designates 13-HETE, 10-HETE, and 7-HETE. The 13-HETE peak on SP-HPLC could be contaminated with 18-HETE, 17-HETE and 16-HETE on its back shoulder (Brash et al., 1995), and other HETEs could also be formed in addition to the bis-HETEs.

N.D., not determined.

Σ designates sum of metabolites; HL, adult human liver; FL, fetal human liver. The recombinant enzymes and microsomes were incubated for 30 min except for CYP1A2 (120 min).


μm, 250 × 4.6 mm) for RP-HPLC and silica (5-μm, 250 × 4.6 mm) for SP-HPLC. The RP-HPLC column was eluted with methanol/water/acetic acid (75:25:0.01) at 1 ml/min, and the SP-HPLC column was eluted with hexane/isopropanol/acetic acid (98:2:0.1) at 0.5 to 1 ml/min. The elution order of HETEs on SP-HPLC was 12-HETE, 15-HETE, 11-HETE, 13-HETE, 18-HETE, 10-HETE, 19-HETE, 7-HETE and 20-HETE.

Three different columns were used for chiral HPLC: silica with (R)-(-)-3,5-dinitrobenzoyl-α-phenylglycine (ionically bonded; 5 μm, 250 × 4.6 mm; Oliw et al., 1990), and Chiralcel OD-H for analysis of 12-HETE and Chiralcel OD-H (5 μm, 250 × 4.6 mm) for analysis of 13-HETE (Brash et al., 1995). These chiral columns were eluted with 0.5%, 2% and 2% isopropanol in hexane, respectively, at 0.5 to 1.0 ml/min. The HPLC columns were connected to a photo diode array detector (Waters 991) and to a radioactivity detector for HPLC (Radiomatic Flo-one 150TR, Packard) using Ultima Flo AP (Packard) as scintillator. The internal standard (13-HODE, 15-HETE) was monitored by UV absorption at 235 nm during HPLC. In addition, large amounts of the bisallylic hydroxy metabolites could be monitored by their end absorption at 205 to 220 nm (Brash et al., 1995).

**Derivatizations and GC-MS analysis.** Trimethylsilyl ethers were prepared by treatment with bis(trimethylsilyl)trifluoroacetamide and pyridine and methyl esters with diazomethane (Oliw et al., 1993) were prepared by treatment with bis(trimethylsilyl)trifluoroacetamide and pyridine and methyl esters with diazomethane (Oliw et al., 1993). A capillary GC (Varian 3100) with a nonpolar column (54 Bylund et al., 1993) and Chiralcel OD-H for analysis of 12-HETE and Chiralcel OD-H (5 μm, 250 × 4.6 mm) for analysis of 13-HETE (Brash et al., 1995). These chiral columns were eluted with 0.5%, 2% and 2% isopropanol in hexane, respectively, at 0.5 to 1.0 ml/min. The HPLC columns were connected to a photo diode array detector (Waters 991) and to a radioactivity detector for HPLC (Radiomatic Flo-one 150TR, Packard) using Ultima Flo AP (Packard) as scintillator. The internal standard (13-HODE, 15-HETE) was monitored by UV absorption at 235 nm during HPLC. In addition, large amounts of the bisallylic hydroxy metabolites could be monitored by their end absorption at 205 to 220 nm (Brash et al., 1995).

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**Other analyses.** Protein was determined as described by Bradford (1976) using γ-globulin as a standard. Testosterone 6β-hydroxylase activity was assayed according to directions of the supplier of [14C]testosterone (Amersham) using TLC on silica plates (20 × 20 cm; Whatman LK6D, Maidstone, UK; eluent CH2Cl2/acetone, 4:1) for analysis. Radioactivity of the TLC plates was determined by a TLC scanner (Berthold Dünschichtsscanner II; Kebo, Stockholm, Sweden). The Rf values were 0.5 and 0.25 for testosterone and 6β-hydroxytestosterone, respectively. O-Dealkylation of ethoxyresorufin and 4′-hydroxylation of diclofenac sodium by human liver microsomes were assayed as described previously (Burke et al., 1985; Leemann et al., 1992).

### Results

**Recombinant human CYP and linoleic acid.** RP-HPLC of metabolites of [14C]18:2n-6, which were formed by NADPH and CYP1A2 (60 pmol, 2 hr) expressed in lymphoblastoid cells and by CYP2C8 (25 pmol, 0.5 hr), CYP2C9 (50 pmol, 0.5 hr) and CYP3A4 (25 pmol, 0.5 hr) expressed in insect cells, are shown in figure 1, A–D. All four enzymes formed a prominent metabolite with an elution time of ~17 to 18 min. This metabolite had the same retention time as [14C]11-HODE formed by liver microsomes of DEX-treated rats (Hörnsten et al., 1996). CYP1A2 and CYP3A4 formed this metabolite, 11-HODE, as a major product. A fifth enzyme, CYP2C19, also formed some 11-HODE (table 1).

In addition to 11-HODE, [14C]labeled compounds eluted with the same retention time as 17-HODE (~21 min), 18-HODE (~23 min) and monoepoxides of 18:2n-6 (~32 min) in all chromatograms (fig. 1) and in experiments with CYP2C19. CYP1A2 appeared to form larger amounts of 17-HODE (fig. 1A) than the other enzymes. Epoxides were partly converted to diols, which eluted after 13 min, by mi-

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**Fig. 1.** RP-HPLC of metabolites formed from [14C]linoleic acid by CYP1A2 (A), CYP2C8 (B), CYP2C9 (C) and CYP3A4 (D). Peak in the chromatograms had the same elution time as [14C]11-HODE and was totally absent in control insect cells and control lymphoblastoid cells. Other products were not characterized, but diols, 17-HODE, 18-HODE, and monoepoxides of 18:2n-6 eluted after ~13, ~21, ~23 and ~30 to 33 min, respectively.
CYP3A4 formed 13-HETE as the major monohydroxy metabolite, followed by 10-HETE and 7-HETE as judged from SP-HPLC (fig. 2C). Biosynthesis of the \( v_1 \) and \( v_2 \) hydroxy metabolites was insignificant. As discussed above, we could not obtain sufficient material for GC-MS analysis by recombinant enzymes. To correct for day-to-day variations in retention times of SP-HPLC, we used UV monitoring of internal standards (13-HODE, 15-HETE). We also analyzed pooled monohydroxy metabolites of \([14C]20:4n-6\) formed by rat liver microsomes of DEX treated rats for comparison (fig. 2D). The major metabolites of the peaks in the chromatogram (marked 7-, 10-, 13-, 19- and 20-HETE in fig. 2D) were previously identified by GC-MS (Hörnsten et al., 1996).

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CYP2C9 formed two major peaks on SP-HPLC (fig. 3A), which eluted after \( \sim 7 \) and \( \sim 10 \) min, respectively, as well as many minor metabolites. The first metabolite had the same retention time as 12-HETE on SP-HPLC (on coinjection; fig. 3B), and it eluted mainly with the 12R-HETE stereoisomer on chiral HPLC (fig. 3C). The second \([14C]20:4n-6\) formed by rat liver microsomes of DEX treated rats for comparison (fig. 2D). The major metabolites of the peaks in the chromatogram (marked 7-, 10-, 13-, 19- and 20-HETE in fig. 2D) were previously identified by GC-MS (Hörnsten et al., 1996).

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CYP2C9 formed 13-HETE as the main and prominent metabolite with relatively little biosynthesis of bisallylic hydroxy metabolites and other HETEs. This enzyme also formed epoxides/diols as major metabolites.

The percent distribution of the arachidonic acid metabolites into epoxides/diols and HETEs by the CYP enzymes with bisallylic hydroxylation activity is summarized in table 2 together with an estimate of the amounts of 20-HETE, 19-HETE and other HETEs (including the bisallylic hydroxy metabolites). Control microsomes without expressed CYP did not significantly metabolize arachidonic acid.

Bisallylic hydroxylation of linoleic and arachidonic acids by fetal and adult human liver microsomes. Fetal liver microsomes converted \([14C]18:2n-6\) to \([14C]11\)-HODE as judged from RP-HPLC (fig. 4A). This metabolite was also identified by GC-MS. The product was formed in similar amounts as 18-HODE and epoxides/diols (table 1). Two different samples of human fetal liver yielded similar results.

\[ \text{R144C of CYP2C9 yielded similar results. CYP2C8 converted} \]
[14C]20:4n-6 was converted by fetal liver microsomes to 20-HETE and 13-HETE as major monohydroxy metabolites by two samples of fetal liver microsomes (table 2). Radiolabeled material with the retention times of 10-HETE, 7-HETE and 19-HETE was also noted on SP-HPLC.

Adult human liver microsomes from three patients (HL37, HL42 and HL46) were also investigated. 18:2n-6 was mainly converted to 18-HODE and diols, and only small amounts of 11-HODE were formed by the three samples (HL37; fig. 4B). However, in the presence of 7,8-benzoflavone (100 μM), the amounts of 11-HODE appeared to increase 2- to 3-fold in the three liver samples (data not shown). The stimulatory effect of 7,8-benzoflavone appeared to be even more striking when [14C]20:4n-6 was used as a substrate. Figure 5, A and B, shows the monohydroxy metabolites of [14C]20:4n-6 formed by human liver microsomes (HL37) with and without 7,8-benzoflavone (0.1 mM) present, respectively. 7,8-Benzoflavone appeared to markedly increase the biosynthesis of 7-HETE and decrease biosynthesis of 20-HETE in comparison with the control incubations. HL42 and HL46 yielded similar results. 7,8-Benzoflavone is a selective inhibitor of CYP1A2 at low concentrations (1–10 μM), whereas high concentrations (100 μM) inhibit many different CYP enzymes but can augment the activity of CYP3A4 (Newton et al., 1995; Yun et al., 1992). A low concentration of 7,8-benzoflavone (5 μM) was found to reduce the biosynthesis of 11-HODE (HL42 and HL46), as discussed below.

**Effect of CYP inhibitors on adult human liver microsomes.** The screening of CYP enzymes indicated that CYP3A4, the CYP2C subfamily and CYP1A2 might contribute to the bisallylic hydroxylation activity of human microsomes. Specific inhibitors of these enzymes were studied.

TAO (10–50 μM) did not affect the bisallylic hydroxylation of three different adult human liver microsomes. TAO is a mechanism-based inhibitor of the CYP3A subfamily, including CYP3A4 (Newton et al., 1995; Ono et al., 1996). To confirm that concentration of TAO was sufficient, we confirmed that 10 μM TAO reduced 6β-hydroxylation of testosterone in these human microsomes by >90% (see Newton et al., 1995). In addition, TAO (1–10 μM) inhibited the biosynthesis of 11-HODE from 18:2n-6 by liver microsomes of DEX-treated rats (see below).

We also investigated the effects of sulfaphenazole (50 μM), an inhibitor of the CYP2C subfamily (except CYP2C19), and two inhibitors of CYP1A2, furafylline (20 μM), a mechanism-based inhibitor and 7,8-benzoflavone (Crespi et al., 1997; Newton et al., 1995; Ono et al., 1996), on the bisallylic hydroxylation of 18:2n-6 by three different samples of adult human liver microsomes (HL37, HL42, HL46).

Sulfaphenazole did not inhibit the bisallylic hydroxylation of linoleic and arachidonic acids, but there appeared to be some reduction in the biosynthesis of epoxides/diols activity, in agreement with studies by Rikkind et al. (1995). We confirmed that drug concentration was sufficient. Sulfaphenazole reduced by >90% the 4′-hydroxylation of ethoxyresorufin by >90% (HL42, HL46).

Furafylline (20 μM; three livers) and 7,8-benzoflavone (5 μM: HL42, HL46) reduced the bisallylic hydroxylation activity on 18:n-6 by 60% and 40% to 50%, respectively. At this concentration both drugs inhibited the O-dealkylation of ethoxyresorufin by >90% (HL42, HL46).

**Effect of CYP inhibitors on fetal liver microsomes.** Fetal liver microsomes contain CYP3A7. TAO is also an inhibitor of CYP3A7 (Hashimoto et al., 1995), but TAO (10 μM) did not reduce the biosynthesis of 11-HODE by fetal liver microsomes. Furafylline (20 μM) did not inhibit the biosynthesis of 11-HODE by fetal liver microsomes.

**Steric analysis of 13-HETE.** The stereochemistry of 13-HETE formed by human liver microsomes and some recombinant CYP was determined by chiral HPLC (Chiralcel OD-H) as described previously (Brash et al., 1995). As a standard, we used 13-HETE formed by liver microsomes of
phenobarbital-treated rats, which was found to contain 60% 13R-HETE (see Brash et al., 1995).

Human and fetal liver microsomes appeared to form 13-HETE with different chirality. Two samples of fetal liver microsomes mainly formed 13R-HETE (75–80%), whereas three samples of adult human liver mainly formed 13S-HETE (73–90%) as judged from chiral HPLC (fig. 6, A, B and D). The results indicated that adult and fetal liver microsomes form 13-HETE by different enzymes.

In the presence of 7,8-benzoflavone (100 μM), adult human livers formed 13-HETE with much less stereospecificity (fig. 6, C and D). As discussed above, 7,8-benzoflavone (100 μM) can augment the enzyme activity of CYP3A4. We found that CYP3A4 formed virtually racemic 13-HETE on Chiralcel OD-H. It therefore seems likely that CYP3A4 may contribute to the increase in bisallylic hydroxylation activity of human liver microsomes in the presence of stimulatory concentrations of 7,8-benzoflavone.

Steric analysis of 13-HETE from CYP1A2 showed that it contained 75% of 13S-HETE and 25% 13R-HETE. CYP1A2 and human liver microsomes thus mainly formed the 13S-antipode of 13-HETE (75% and 73–90%, respectively). In some analysis of 13-HETE methyl ester (CYP1A2, human liver microsomes) on chiral HPLC, a peak of radioactivity some analysis of 13-HETE methyl ester (CYP1A2, human liver microsomes) on chiral HPLC, a peak of radioactivity

Liver microsomes of Fischer rats. 11-HODE was formed as a minor metabolite of 18:2n-6 by liver microsomes of untreated rats. Starvation for 48 hr did not appear to increase its biosynthesis (n = 3), whereas treatment with starvation and acetone has been found to do so previously (Hörnsten et al., 1996). ERY treatment appeared to induce the biosynthesis of 11-HODE —2-fold, and DEX treatment induced biosynthesis by —10-fold. We confirmed that both treatments strongly (5–20-fold) induced the 6β-hydroxylation of testosterone in liver. We also found that DEX treatment increased bisallylic hydroxylation activity in liver microsomes of both sexes. As expected, there appeared to be an increased bisallylic hydroxylation of 20:4n-6 after treatment with DEX. —2-fold for 13-HETE and 10-HETE in some experiments and —4-fold for 7-HETE (data not shown). 13-HETE prepared by liver microsomes of DEX-treated rats was found to be racemic by chiral HPLC.

DEX and ERY are known to induce the CYP3A subfamily, particularly CYP3A1 and closely related enzymes, in the rat liver (Daujat et al., 1991). CYP3A1 was therefore an obvious candidate with bisallylic hydroxylation activity. This possibility was assessed by TAO, an inhibitor of the CYP3A subfamily (Newton et al., 1995; Ono et al., 1996), and by inhibitory antibodies against CYP3A1. TAO dose-dependently reduced the biosynthesis of 11-HODE with an ED50 of 1 μM (fig. 7). TAO caused 85% inhibition at 10 μM. TAO (10 μM) also appeared to reduce the bisallylic hydroxylation of 20:4n-6 to 13-HETE and 7-HETE by >50%. Finally, polyclonal antibodies against CYP3A1 (1 mg/ml) were found to inhibit almost completely the bisallylic hydroxylation of [14C]18:2n-6 by liver microsomes of DEX-treated rats, whereas the control incubation, which contained IgG (1 mg/ml) instead, formed [14C]11-HODE (data not shown).

GC-MS analysis and steric analysis of 11-HODE. 11-HODE was identified from an incubation with fetal liver microsomes with 18:2n-6 and NADPH. The C-value and the mass spectrum were as reported previously (Hamberg et al., 1992). The stereochemistry of 11-HODE formed by liver microsomes of DEX-treated rats was found to be nearly racemic (58% R). 11-HODE is also formed by liver microsomes of phenobarbital-treated rats, and in this case the result was almost identical, 59% 11R-HODE (Oliw et al., 1993).

Discussion

The present study focuses on bisallylic hydroxylation and hydroxylation with double bond migration of polyunsaturated fatty acids. Polynsaturated fatty acids are also metabolized to epoxides and to ω1 and ω2 hydroxy metabolites as major products (Capdevila et al., 1995; Oliw, 1994; Oliw et al., 1996). These reactions can be catalyzed by many different CYP enzymes and have been studied extensively, whereas the present work is the first systematic study of human recombinant CYP with bisallylic hydroxylase activity.

Human recombinant enzymes. We report three new findings. Five of 10 human recombinant CYP, viz., CYP1A2, CYP3A4, CYP2C8, CYP2C19 and two isoforms of CYP2C9 (Arg144 and Cys144), were found to metabolize 18:2n-6 to 11-HODE and 20:4n-6 to 13-HETE. CYP1A2 and CYP3A4 also hydroxylated the two other bisallylic carbons of 20:4n-6, although 13-HETE was clearly the main bisallylic hydroxy

![Fig. 6](image-url)  
Fig. 6. Steric analysis of 13-HETE methyl ester obtained from incubations with fetal and adult human liver microsomes using chiral HPLC. A, 13-HETE obtained from fetal liver microsomes. B, 13-HETE from adult human liver microsomes (HL46). C, 13-HETE from adult human liver microsomes (HL42) incubated with 100 μM 7,8-benzoflavone. D, 13-HETE from liver microsomes (HL42) incubated without 7,8-benzoflavone. Before chiral HPLC, 13-HETE was purified by RP- and SP-HPLC.

![Fig. 7](image-url)  
Fig. 7. Effect of troleandomycin on the biosynthesis of [14C]11-HODE from [14C]linoleic acid by liver microsomes of DEX-treated rats.
metabolite. CYP2C9 and CYP2C8 yielded a different pattern. The former also oxidized 20:4n-6 to 12R-HETE as a main metabolite and to small amounts of 15-HETE, 11-HETE and 10-HETE. The latter formed 13-HETE along with 15-HETE, 11-HETE and small amounts of 10-HETE. CYP1A2 and CYP3A4 can thus catalyze bisallylic hydroxylations, whereas CYP2C8 and CYP2C9 also can form cis-trans conjugated HETEs, presumably by hydroxylation with double bond migration. Some of the main hydroxy metabolites formed by these five enzymes are summarized in figure 8.

Our second finding, which is based on specific inhibitors and steric analysis of 13-HETE, is that CYP1A2 could contribute to the bisallylic hydroxylation activity of adult human liver microsomes. CYP1A2, CYP3A4 and the CYP2C subfamily together make up ~60% of the CYP enzymes of human liver, but there are large interindividual variations (Guengerich, 1995). In view of the bisallylic hydroxylation activity of the recombinant enzymes discussed above, we expected that specific inhibitors of CYP1A2, the CYP2C subfamily and CYP3A4 would reduce the bisallylic hydroxylation activity of human liver microsomes. Only inhibitors of CYP1A2 (furafylline, 7,8-benzoflavone) appeared to do so, whereas inhibitors of the CYP3A subfamily (TAO) and the CYP2C subfamily (sulfaphenazole) were without effects.

Furafylline and low concentrations of 7,8-benzoflavone are known to inhibit CYP1A2 with selectivity (Newton et al., 1995; Ono et al., 1996). Both drugs reduced the bisallylic hydroxylation of 18:2n-6 by 60% and 40%, respectively. In addition, both CYP1A2 and adult human liver microsomes mainly formed the 13S stereoisomer of 13-HETE. The selective inhibitors and the steric analysis suggested that CYP1A2 could contribute to the bisallylic hydroxylation activity of adult human liver microsomes in vitro, but other CYP enzymes might also contribute.

Our third finding is that fetal liver, microsomes had a prominent bisallylic hydroxylation activity on 18:2n-6 and 20:4n-6 in comparison with the epoxygenation and hydroxylation of these fatty acids. Interestingly, the chirality of 13-HETE formed by fetal and adult liver microsomes differed. 13-HETE from fetal liver contained >70% of the R stereoisomer, and that from adult liver contained >70% of the S stereoisomer. This finding suggested that fetal and adult liver microsomes formed 13-HETE by different enzymes. Neither TAO nor furafylline reduced the bisallylic hydroxylation activity of fetal liver microsomes.

The lack of effect of TAO on the bisallylic hydroxylation activity of adult and fetal human microsomes was unexpected. CYP3A4 and CYP3A7 are quantitatively major enzymes in adult and fetal human liver, respectively (Guengerich, 1995). We also confirmed that our human adult liver microsomes catalyzed a typical CYP3A4 reaction, the 6β-hydroxylation of testosterone. The difference between human adult liver microsomes and recombinant CYP3A4 could be related to differences in coupling between cytochrome P450 reductase and CYP3A4. The recombinant CYP3A4 was coexpressed with NADPH-P450 reductase. The enzymatic activity of CYP3A4 in microsomes can be augmented by 0.1 mM 7,8-benzoflavone (Berthou et al., 1994; Schwab et al., 1988; Yun et al., 1992). The mechanism is unknown, but the drug may facilitate electron flow from the reductase to the monooxygenase. We observed that 7,8-benzoflavone (0.1 mM) could increase the bisallylic hydroxylation activity of adult human liver microsomes. 13-HETE, which was formed in the presence of 7,8-benzoflavone, contained more 13R-HETE and was thus formed with less stereospecificity. CYP3A4 biosynthesized racemic 13-HETE. It therefore seems likely that 7,8-benzoflavone stimulated the bisallylic hydroxylation activity of CYP3A4 in the microsomes.

**Rat liver enzymes.** We extended our studies on induction of bisallylic hydroxylation activity of rat liver microsomes (Hörnsten et al., 1996). Treatment with two different inducers of CYP3A1, DEX and ERY, increased the bisallylic hydroxylation activity in rats of both sexes. Furthermore, TAO and polyclonal antibodies against CYP3A1 strongly inhibited this enzymatic reaction. It therefore seems likely that the principal bisallylic hydroxylation activity of liver microsomes of rats treated with DEX and ERY is associated with CYP3A1 or any of its closely related isozymes (Kirita and Matsubara, 1993; Komori and Oda, 1994; Pereira and Lechner, 1995). Phenobarbital treatment can also induce CYP3A1 (Larsen and Jefcoate, 1995). This may contribute to the fact that liver microsomes of DEX and phenobarbital-treated rats formed 11-HODE with the same chirality (58–59% 11R-HETE).

**Hydroxylation mechanisms.** Bisallylic hydroxy metabolites are likely formed, in analogy with other hydroxylations, by hydrogen abstraction by the heme ferryl oxygen and “oxygen rebound” (Ortiz de Montellano, 1995). Oxygen is inserted with retention of configuration during biosynthesis of 11-HODE (Oliw et al., 1993). The bisallylic carbon must thus be positioned near the active center of the enzyme. CYP1A2 and CYP3A4 apparently allowed all three bisallylic

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**Fig. 8.** Overview of bisallylic hydroxylations and hydroxylation with double bond migration. A, Bisallylic hydroxylation of linoleic acid to 11-HODE (1). B, Bisallylic hydroxylation of arachidonic acid to 7-HETE (2), 10-HETE (3) and 13-HETE (4). C, Hydroxylation with double bond migration of arachidonic acid to 12R-HETE (5) and bisallylic hydroxylation to 13-HETE (6). In addition, all five recombinant enzymes also formed epoxides/diols, and many of them formed hydroxylated carbons of the ω side chain.
carbons of 20:4n-6 to interact with the heme iron. CYP2C9 and CYP2C8 differed; they formed 13-HETE as a main hydroxy metabolite, small amounts of 10-HETE and relatively large amounts of certain cis-trans conjugated HETEs (CYP2C9, mainly 12R-HETE; CYP2C8, mainly 11-HETE and 15-HETE). Both enzymes can abstract a hydrogen from C13 and C10 and insert oxygen at these carbons. It seems likely that the double bond may also migrate. After hydrogen abstraction at C13 by CYP2C8, oxygen could be inserted at C11 and C15, yielding 11-HETE and 15-HETE. After hydrogen abstraction at C10 by CYP2C9, oxygen could be inserted at C12, yielding 12R-HETE. Linoleic acid can undergo similar transformations to 11R,S-HODE, 9R-HODE and 13R-HODE by rat liver microsomes (Oliw et al., 1993).

To abstract a hydrogen from a bisallylic carbon requires less energy than from other carbons of the fatty acid (Porter et al., 1995). Alternatively, the reactive ferryl oxygen may to oxidize the double bonds (epoxidation) adjacent to the bisallylic carbon. CYP2C9, for example, formed 14(15)epoxyeicosatrienoic acid as the main metabolite of 20:4n-6. All five CYP enzymes, which catalyzed bisallylic hydroxylations, were also found to epoxidize double bonds. It therefore seems likely that epoxidation and bisallylic hydroxylations could be connected. However, CYP102 of Bacillus megaterium has been reported to have arachidonate o6 epoxygenase activity without formation of bisallylic or cis-trans conjugated HETEs (Capdevila et al., 1996). All epoxygenases therefore may not catalyze bisallylic hydroxylations.

12R-HETE and 15R-HETE. CYP2C9 synthesized relatively large amounts of 12-HETE and mainly the 12R anti-pode. 12R-HETE is also a well known metabolite formed by human and rat liver microsomes (McGiff, 1991). The enantiomeric purity is \( \approx 80\% \) when extracted at acidic pH and >90% at neutral pH. The chirality of 12-HETE suggests that CYP2C9 may contribute to biosynthesis of 12R-HETE by adult human liver microsomes. CYP2C9 could be useful as a model enzyme for studying this reaction in detail. CYP2C9 and its allelic form R144C can hydroxylate a large number of drugs (e.g., tolbutamide, phenytoin, diclofenac sodium and many other nonsteroidal anti-inflammatory drugs; Goldstein and de Morais, 1994), but none of these substrates seems to be subject to hydroxylation with double bond migration.

Adult human liver microsomes also form 15-HETE, which consists predominantly of the 15R antipode (Oliw, 1993). The present work demonstrated that adult human liver microsomes mainly formed 13S-HETE. This is an interesting analogy with oxygenation of 18:2n-6 by rat liver microsomes. Biosynthesis of 11S-HODE and 13R-HODE from stereospecifically deuterated 18:2n-6 occurred by abstraction of the pro-S hydrogen at C11 of 18:2n-6 and suprafacial oxygen insertion (Oliw et al., 1993). From the biosynthesis of 12R-HETE and 10-HETE by CYP2C9, it clearly will be of interest to determine whether this occurs with formation of the S antipode of 10-HETE.

Biological significance of CYP metabolites. A biological role of epoxides of 20:4n-6, 20-HETE and 19-HETE has been implicated in different organ systems, hormonal signal-

References


