ALIPHATIC HYDROXYLATION BY HIGHLY PURIFIED LIVER MICROSONAL CYTOCHROME P-450. EVIDENCE FOR A CARBON RADICAL INTERMEDIATE

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Summary: The oxidation of norbornane by a reconstituted liver cytochrome P-450 system affords exo- and endo-2-norborneol in a ratio of 3.4:1. The ratio of these products was found to be 0.76:1 when exo,exo,exo,exo-2,3,5,6-tetradueteronorbornane was oxidized. Analysis of the mass spectra of the products from the deuterated hydrocarbon showed that 25% of the exo-norborneol contained four deuterium atoms whereas 9% of the endo-norborneol contained three deuterium atoms. These results, which indicate a very large isotope effect (kH/kD = 11.5±1) and a significant amount of epimerization for the hydroxylation of norbornane by cytochrome P-450, suggest an initial hydrogen abstraction to give a carbon radical intermediate.

The heme-containing mixed function oxidase of liver microsomes, cytochrome P-450, has been the subject of much investigation because of its ability to catalyze epoxidation or hydroxylation of a wide variety of organic compounds (cf. 1-3). Several lines of evidence suggest that the reactive oxygen intermediate is a higher valent iron-oxo species equivalent to [FeO]3+ (4-7). The mechanism by which this proposed oxo-species transfers the equivalent of atomic oxygen to the substrate has remained obscure. Recent observations in one of our laboratories (8-10) have shown that simple iron-peroxide systems effect hydroxylation of saturated carbon centers by a mechanism which is similar

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to that of P-450 in several ways. We report here evidence that, like the model system, purified rabbit liver microsomal cytochrome P-450 (phenobarbital induced P-450\textsubscript{LM2}) catalyzes aliphatic hydroxylation by hydrogen abstraction to give a carbon radical intermediate.

**Materials and Methods.** In a typical experiment, the reaction mixture contained electrophoretically homogeneous P-450\textsubscript{LM2} (2 nmol), NADPH-cytochrome P-450 reductase (1.5 nmol), dilauroyl glyceryl-3-phosphorylcholine (0.1 mg), norbornane (2 \textmu mol), and sodium phosphate buffer, pH 7.4 (100 \textmu mol). NADPH (2 \textmu mol) was added in one portion (final volume 1 ml) and the reaction mixture was allowed to stand at room temperature for 30 minutes. The mixture was extracted with 2 ml methylene chloride and analyzed for reaction products by gc on a 5-ft, 3\% STAP column at 80°, 40 ml/min (exo-2-norborneol, 10 min; endo-2-norborneol, 11 min; 7-norborneol, 13 min; 2-norbornanone, 5 min; 7-norbornanone, 4.5 min). Products were identified by comparison of retention times and mass spectral fragmentations with those of authentic samples. Turnover numbers (nmol/min/nmol P-450\textsubscript{LM2}), determined by measuring the rate of disappearance of NADPH at 340 nm, were 73 for benzphetamine, 50 for norbornane and 45 for \textsuperscript{2}.

Trimethylsilyl ethers of \textsuperscript{1} and \textsuperscript{2} were prepared by standard techniques using O,N-bis-trimethylsilylacetamide. The silyl ethers of \textsuperscript{1} and \textsuperscript{2} exhibited base peaks corresponding to M\textsuperscript{+}-CH\textsubscript{3} which could be analyzed for deuterium content with confidence (\textsuperscript{1} and \textsuperscript{2-OTMS from \textsuperscript{3}}, \textfrac{d_3}{d_4} = 0.615 from the ratio of M\textsuperscript{+}-CH\textsubscript{3} intensities). No conditions were found which would allow gas chromatographic resolution of the stereoisomers.

**Results and Discussion.** The hydroxylation of alkanes by a reconstituted cytochrome P-450\textsubscript{LM2} system has been previously described (4,11-14). In the present study (15), norbornane was shown to be a substrate for this system and to produce only \textit{exo-} and \textit{endo-}2-norborneol (\textsuperscript{1} and \textsuperscript{2}) in a ratio of 3.4:1. In contrast, \textit{exo,exo,exo,exo-}2,3,5,6-tetradenueronorborne (\textsuperscript{3}) (16,17) gave a ratio of 0.76:1. Both starting materials produced similar overall yields of product at nearly identical rates. The variation in stereoisomer ratio with deuterium substitution is best interpreted as a result of a significant kinetic isotope effect (\textit{k_H/k_D}) and some degree of intrinsic stereospecificity in the reaction.
Table 1. Mass Spectra of exo- and endo-Norborneols [M⁺-H₂O(HOD)]a

<table>
<thead>
<tr>
<th>m/e</th>
<th>93</th>
<th>94</th>
<th>95</th>
<th>96</th>
<th>97</th>
<th>98</th>
<th>99</th>
<th>% d₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 from 3</td>
<td></td>
<td>10.6</td>
<td>33.7</td>
<td>100</td>
<td>41.7</td>
<td>9.7</td>
<td>75%</td>
<td>d₃,25%d₄</td>
</tr>
<tr>
<td>calcd for d₃/d₄=3.0</td>
<td></td>
<td>6.6</td>
<td>33.3</td>
<td>100</td>
<td>41.7</td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 from 3</td>
<td></td>
<td>4.5</td>
<td>9.5</td>
<td>44.4</td>
<td>100</td>
<td>11.2</td>
<td>9%</td>
<td>d₃,91%d₄</td>
</tr>
<tr>
<td>calcd for d₃/d₄=0.10</td>
<td></td>
<td>0.7</td>
<td>10.8</td>
<td>44.4</td>
<td>100</td>
<td>12.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>exo-4b</td>
<td></td>
<td>6.9</td>
<td>35.8</td>
<td>100</td>
<td>14.3</td>
<td></td>
<td>&gt;95%</td>
<td>d₂c</td>
</tr>
<tr>
<td>endo-4b</td>
<td></td>
<td>6.9</td>
<td>35.8</td>
<td>100</td>
<td>14.3</td>
<td></td>
<td>&gt;95%</td>
<td>d₂c</td>
</tr>
<tr>
<td>exo-norborneol</td>
<td></td>
<td>6.8</td>
<td>100</td>
<td>10.7</td>
<td></td>
<td>100%</td>
<td>d₀</td>
<td></td>
</tr>
<tr>
<td>endo-norborneol</td>
<td></td>
<td>6.8</td>
<td>100</td>
<td>10.7</td>
<td></td>
<td>100%</td>
<td>d₀</td>
<td></td>
</tr>
</tbody>
</table>

aAll reported values are typical of multiple spectra (10-15) of duplicate samples. Typical standard deviations were ±1%.
bPrepared by hydroboration-oxidation of norbornadiene and reduction with D₂, cf. ref. 16.
cFrom the mass spectrum of the corresponding silyl ethers.

The hydroxylations mediated by cytochrome P-450 generally proceed with net retention of configuration at the oxidized carbon center. The degree to which this is so for the P-450Lm2 catalyzed hydroxylation of norbornane is readily apparent upon examination of the mass spectra of 1 and 2 derived from 3 (Table 1). Qualitatively, the presence of a large peak at m/e 98 (M⁺d₄-H₂O) in the mass spectrum of 1 from 3 requires a significant component of d₄ material in the exo-norborneol product (exo-6) and, accordingly, a non-stereospecific hydroxylation.

A more accurate measure of the deuterium content of the exo- and endo-norborneols formed (1 and 2) can be derived from the prominent M⁺-H₂O(HOD) spectral pattern of these compounds. Studies with various labeled norborneols have shown conclusively
that the exo and endo-stereoisomers have identical fragmentation patterns and that negligible loss of deuterium occurs from position 2 or 3 upon loss of water from the parent ion (18-21). Accordingly, the tri- or tetradeuterated derivatives \( \tilde{5} \) and \( \tilde{6} \) are expected to have identical M-H\(_2\)O(HOD) spectral patterns displaced by 1 and 2 nominal mass units from that of \( \tilde{4} \).

Thus, the deuterium content of \( \tilde{5} \) produced from \( \tilde{3} \) can be determined to be 75\% d\(_3\) and 25\% d\(_4\) while \( \tilde{2} \) produced from \( \tilde{3} \) is 91\% d\(_4\) and 9\% d\(_3\). Observed and calculated mass spectra for these deuterium distributions are compared in Table 1. The net ratio of norborneol-d\(_3\) to norborneol-d\(_4\) determined in this way (d\(_3\)/d\(_4\) = 6.0) was corroborated by that measured independently from the corresponding silyl ethers (d\(_3\)/d\(_4\) = .615).

The d\(_4\) alcohol in \( \tilde{1} \) results from an 18\% endo \rightarrow exo inversion component in the course of the hydroxylation while the d\(_3\) alcohol in \( \tilde{2} \) results from a 14\% exo \rightarrow endo crossover. Thus, aliphatic hydroxylation by P-450\(_{LM2}\) is not nearly as stereo-specific as has been commonly assumed, at least with this substrate. Further, the ratio of \( \tilde{1} \) to \( \tilde{2} \) derived from norbornane (3.4:1) must be corrected for this crossover and the intrinsic relative reactivities of the exo- and endo-hydrogens at C-2 can be calculated according to equation (1).

\[
\frac{1}{2} = \frac{0.86 k_{\text{exo}} + 0.18 k_{\text{endo}}}{0.82 k_{\text{endo}} + 0.14 k_{\text{exo}}} = 3.4; \quad \frac{k_{\text{exo}}}{k_{\text{endo}}} = 7.0 \quad (1)
\]
The kinetic hydrogen isotope effect \( \frac{k_H}{k_D} \) for hydroxylation is related to \( \frac{k_{\text{exo}}}{k_{\text{endo}}} \) and the deuterium content of all products from \( \gamma \) (\( \frac{d_3}{d_4} \)) according to equation (2).

\[
\frac{k_H}{k_D} = \left( \frac{k_{\text{exo}}}{k_{\text{endo}}} \right) \frac{d_4}{d_3}
\]

(2)

The deuterium content in the products from \( \gamma \) (\( \frac{d_3}{d_4} \)), whether calculated from the alcohol mass spectra or determined from the silyl ether spectra, requires that the isotope effect for \text{exo}-hydrogen abstraction in \( \gamma \) be at least 5.7. Accounting for the stereochemical crossover noted above, the actual isotope effect is found to be 11.5±1. This large value is similar to those observed for alkane oxidations by well characterized oxo complexes of manganese and chromium (22,23), and similar also to \text{intramolecular} hydrogen isotope effects observed by Foster (24) and Hjelmeland (25) for liver microsomal preparations. In contrast, intermolecular hydrogen isotope effects for hydroxylations by liver microsomes are usually less than 2 (cf. 26,27).

The large isotope effect and the significant amount of epimerization are consistent with homolytic hydrogen abstraction of the C-2 hydrogen by P-450\textsubscript{LM2} as the site-determining step, leading to an intermediate carbon radical which undergoes partial epimerization in the enzyme-substrate cage (Scheme I). It is unlikely that a free carbonium ion is ever formed in this process since the participation of the 2-norbornyl cation should strongly mitigate against \text{exo} + \text{endo} crossover. This process, hydrogen abstraction followed by formal ligand transfer and accompanied by some loss of stereochemistry, is reminiscent of the mechanism for the alkane oxidation by iron-peroxide systems in solution (8).
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References