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ALIPHATIC HYDROXYLATION BY HIGHLY PURIFIED LIVER MICROSOMAL 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 (k_H/k_D = 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$_{LM2}$) 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$_{LM2}$ (2 nmol), NADPH-cytochrome P-450 reductase (1.5 nmol), dilauroyl glyceryl-3-phosphorylcholine (0.1 mg), norbornane (2 µmol), and sodium phosphate buffer, pH 7.4 (100 µmol). NADPH (2 µmol) 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 glc 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$_{LM2}$), determined by measuring the rate of disappearance of NADPH at 340 nm, were 73 for benzphetamine, 50 for norbornane and 45 for 3.

Trimethylsilyl ethers of 1 and 2 were prepared by standard techniques using O,N-bis-trimethylsilylacetamide. The silyl ethers of 1 and 2 exhibited base peaks corresponding to M^+-CH_3 which could be analyzed for deuterium content with confidence (1 and 2-OTMS from 3, d_3/d_4 = 0.615 from the ratio of M^+-CH_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$_{LM2}$ 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 exo- and endo-2-norborneol (1 and 2) in a ratio of 3.4:1. In contrast, exo,exo,exo,exo-2,3,5,6-tetradeuteronorbornane (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 (k_H/k_D) and some degree of intrinsic stereospecificity in the reaction.

Table 1. Mass Spectra of exo- and endo-Norborneols [M^+-H_2O(HOD)][a]

m/e	93	94	95	96	97	98	99	% d_n
1 from 3			10.6	33.7	100	41.7	9.7	75% d_3, 25% d_4
calcd for d_3/d_4=3.0			6.6	33.3	100	41.7	4.6	-- --
2 from 3			4.5	9.5	44.4	100	11.2	9% d_3, 91% d_4
calcd for d_3/d_4=0.10			0.7	10.8	44.4	100	12.6	-- --
exo-4[b]		6.9	35.8	100	14.3			>95% d_2[c]
endo-4[b]		6.9	35.8	100	14.3			>95% d_2[c]
exo-norborneol	6.8	100	10.7					100% d_o
endo-norborneol	6.8	100	10.7					100% d_o

[a]All reported values are typical of multiple spectra (10-15) of duplicate samples. Typical standard deviations were ±1%.

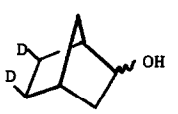
[b]Prepared by hydroboration-oxidation of norbornadiene and reduction with D_2, cf. ref. 16.

[c]From the mass spectrum of the corresponding silyl ethers.

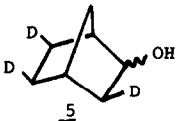
The hydroxylations mediated by cytochrome P-450 generally proceed with net retention of configuration at the oxidized carbon center. The degree to which this is so for the $P\text{-}450_{LM2}$-catalyzed hydroxylation of norbornane is readily apparent upon examination of the mass spectra of 1 and 2 derived from 3 (Table 1). Qualitatively, the presence of a large peak at m/e 98 ($M^+d_4-H_2O$) in the mass spectrum of 1 from 3 requires a significant component of d_4 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^+-H_2O(HOD) spectral pattern of these compounds. Studies with various labeled norborneols have shown conclusively

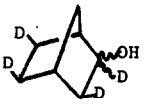
that the exo and endo-stereoisomers have identical fragmentation patterns and that negligible loss of deuterium occurs from position 2 or 3 upon loss of water from the parent ion (18-21). Accordingly, the tri- or tetradeuterated derivatives 5 and 6 are expected to have identical M-H_2O(HOD) spectral patterns displaced by 1 and 2 nominal mass units from that of 4.



4



5



6

Thus, the deuterium content of 1 produced from 3 can be determined to be 75% d_3 and 25% d_4 while 2 produced from 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 1 results from an 18% endo → exo inversion component in the course of the hydroxylation while the d_3 alcohol in 2 results from a 14% exo → endo crossover. Thus, aliphatic hydroxylation by P-450_{LM2} is not nearly as stereospecific as has been commonly assumed, at least with this substrate. Further, the ratio of 1 to 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).

$$1/2 = \frac{0.86\ k_{exo} + 0.18\ k_{endo}}{0.82\ k_{endo} + 0.14\ k_{exo}} = 3.4; \quad \frac{k_{exo}}{k_{endo}} = 7.0 \qquad (1)$$

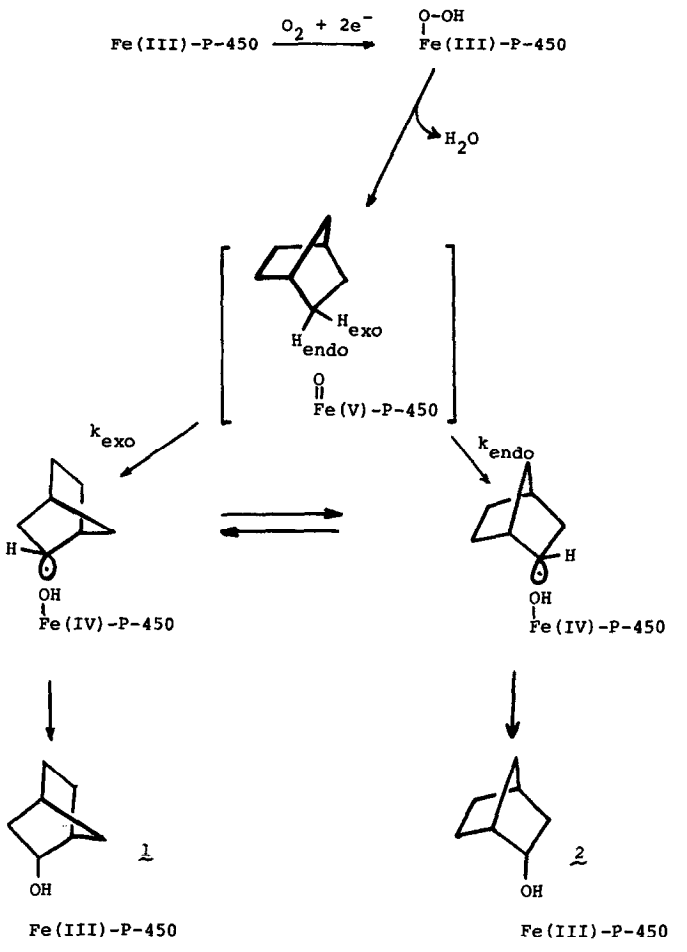
The kinetic hydrogen isotope effect (k_H/k_D) for hydroxylation is related to $k_{\underline{exo}}/k_{\underline{endo}}$ and the deuterium content of all products from $\underset{\sim}{3}$ ($\underline{d}_n$) according to equation (2).

$$k_H/k_D = (k_{\underline{exo}}/k_{\underline{endo}})(\underline{d}_4/\underline{d}_3) \qquad (2)$$

The deuterium content in the products from $\underset{\sim}{3}$ ($\underline{d}_3/\underline{d}_4$), whether calculated from the alcohol mass spectra or determined from the silyl ether spectra, requires that the isotope effect for exo-hydrogen abstraction in $\underset{\sim}{3}$ 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 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 $P\text{-}450_{LM2}$ 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 exo → 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).

Scheme I



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