and DCl (Aldrich) 99% pure. KOD solutions were prepared by dissolving KOH in D_2O. Mixed isotopic waters were prepared gravimetrically, and the mole fraction of deuterium was checked by NMR with the method of Schowen.[21b] Isotopic dilution from buffers was taken into account when preparing reaction solutions; it always amounted to less than 1%.

pH and pK_a Measurements. An Orion 611 digital pH meter with a Corning glass electrode (No. 476022) and a Beckman reference electrode (No. 39402) was used, with the solutions being thermostated at 25 °C. For the stopped-flow runs the pH was measured in mock-mixing experiments. In D_2O pD was obtained as pD = pH_{obsd} + 0.40[44] with pH_{obsd} being the value read from the pH meter. Calibration curves of pL - pH_{obsd} vs. mole fraction of D_2O (n) in H_2O/D_2O mixtures were obtained by measuring pH_{obsd} of 0.002 M LCl solutions. Our calibration curve was in excellent agreement with that of Glasoe and Long.[44]

pK_a values for 5-benzyl and 5-(1-phenylethyl) Meldrum's acid in H_2O and D_2O were measured by classical spectrophotometric procedures at 272 or 273 nm, while the pK_a values of morpholine and glycinamide in D_2O were determined potentiometrically.

Kinetic Measurements. The rates of the reactions of 5-benzyl and 5-(1-phenylethyl) Meldrum's acid were measured in a Durrum stopped-flow apparatus with computerized data analysis while all other kinetic measurements were carried out with a Perkin Elmer 559A double beam spectrophotometer. The k_{obsd} values reported in Tables S1-S6[17] are in most cases the average of several runs.

Appendix

Derivation of Equations 16 and 17. According to eq 6 $k_1^{BL}(n)$ can be expressed by eq 19 and $k_L(n)$ by eq 20. Since the buffer ratio, $R = [B]/[BL^+]$, is kept constant in all H_2O/D_2O mixtures,

$$k_1^{BL}(n) = \left[1 + \frac{a_{L^+}}{K_a^{\pm}}\right]_n \text{slope}\,(n) \tag{19}$$

$$k_L(n) = \left[1 + \frac{a_{L^+}}{K_a^{\pm}}\right]_n \frac{\text{intercept}\,(n)}{a_{L^+}} \tag{20}$$

(44) Glasoe, P. K.; Long, F. A. *J. Phys. Chem.* **1960**, *64*, 188.

we can express a_{L^+} as K_a^{BL}/R with K_a^{BL} being the acid dissociation constant of BL^+. Equations 19 and 20 thus become

$$k_1^{BL}(n) = \left[1 + \frac{K_a^{BL}}{K_a^{\pm}}R\right]_n \text{slope}\,(n) \tag{21}$$

$$k_L(n) = \left[1 + \frac{K_a^{BL}}{K_a^{\pm}}R\right]_n \frac{\text{intercept}\,(n)}{K_a^{BL}(n)}R \tag{22}$$

If we now assume that $K_a^{BL}/K_a^{\pm}$ is independent of n, eq 23 and 24 follow from eq 21 and 22, respectively.

$$\frac{k_1^{BL}(n)}{k_1^{BL}(n=0)} = \frac{\text{slope}\,(n)}{\text{slope}\,(n=0)} \quad (23) = (16)$$

$$\frac{k_L(n)}{k_L(n=0)} = \frac{\text{intercept}\,(n)}{\text{intercept}\,(n=0)}\frac{K_a^{BL}(n=0)}{K_a^{BL}(n)} \tag{24}$$

By combining eq 15 and eq 24 we deduce eq 25, and assuming that $k_L = k_i/K_a^+$

$$\frac{k_i(n)}{k_i(n=0)} = \frac{k_L(n)}{k_L(n=0)}\frac{K_a^{\pm}(n)}{K_a^{\pm}(n=0)} = \frac{\text{intercept}\,(n)}{\text{intercept}\,(n=0)}\frac{K_a^{BL}(n=0)}{K_a^{BL}(n)}\frac{K_a^{\pm}(n)}{K_a^{\pm}(n=0)} \tag{25}$$

Using again the assumption that $K_a^{BL}/K_a^{\pm}$ is independent of n simplifies eq 25 to eq 17.

Acknowledgment. This research was supported by Grant CHE-8315374 from the National Science Foundation.

Supplementary Material Available: All observed pseudo-first-order rate constants, Tables S1-S6 (6 pages). Ordering information is given on any current masthead page.

Timing of the Radical Recombination Step in Cytochrome P-450 Catalysis with Ring-Strained Probes

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Abstract: Nortricyclane, methylcyclopropane, and bicyclo[2.1.0]pentane have been used to probe the catalytic mechanism of microsomal cytochrome P-450. Nortricyclane is oxidized by rat liver microsomes to nortricyclanol without the detectable formation of norborn-5-en-2-ol. Methylcyclopropane is similarly oxidized to cyclopropylmethanol without the detectable formation of 3-buten-1-ol or cyclobutanol. The radical pair in the hydroxylation reaction therefore collapses faster than the cyclopropylmethyl radical rearranges (1×10^8 s^{-1}). In contrast, microsomal oxidation of bicyclo[2.1.0]pentane yields approximately a 7:1 mixture of *endo*-2-hydroxybicyclo[2.1.0]pentane *and* 3-cyclopenten-1-ol. Deuterium labeling studies indicate the endo hydrogen is predominantly or exclusively removed from, and the hydroxyl group is delivered to, the endo face in both the rearranged and unrearranged products. The results indicate that a radical pair is formed in P-450-catalyzed hydroxylations that collapses with stereochemical specificity at a rate in excess of 1×10^9 s^{-1}.

Cytochrome P-450 enzymes reductively activate molecular oxygen to a ferryl-bound species that inserts into unactivated carbon-hydrogen bonds.[1] The product of the reaction with a hydrocarbon is usually the corresponding alcohol. These reactions were first thought to occur by a concerted mechanism, but recent evidence favors a nonconcerted mechanism in which hydrogen abstraction by the activated oxygen is followed by rapid collapse of the resulting radical or ion pair to give the hydroxylated product. Three lines of experimental evidence argue for a nonconcerted

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mechanism: (a) the hydroxylations of norbornane by rabbit liver cytochrome P-450[2] and camphor by P-450$_{cam}$[3] proceed with loss of stereochemistry, (b) the hydroxylations of these and other substrates are subject to large *internal* isotope effects,[2-4] and (c) regiochemical scrambling is observed during the allylic hydroxylation of some substrates.[5,6] This scrambling presumably reflects delivery of the activated hydroxyl group to the two ends of allylically delocalized intermediates. These results provide strong support for a nonconcerted process but afford relatively little information on the reaction intermediate or the nature of the recombination process.

Radical "clock" substrates[7] have been used in efforts to confirm the involvement and estimate the lifetimes of radical intermediates in hydrocarbon hydroxylations. The cyclopropylmethyl group is favored for such studies because the radical derived from it rearranges to the 3-butenyl radical fast enough ($k = 1.3 \times 10^8$ s^{-1} at 25 °C) to make the rearrangement competitive with most radical-quenching reactions.[7] However, the cytochrome P-450 catalyzed hydroxylations of norcarane,[8] pericyclocamphanone,[9] and 5,6-methanocholesterol,[10] each of which can be viewed as involving a substituted cyclopropylmethane, occur without carbon framework rearrangements. These hydroxylations therefore do not involve radical intermediates or occur so rapidly that the rearrangement fails to compete with capture of the carbon radical by the activated oxygen. The collapse of radical pairs at rates that compete with rearrangement of the cyclopropylmethyl radical is not without precedent in the chemical literature.[11] The timing of radical reaction rates is particularly difficult if the cyclopropylmethyl function is part of a rigid framework because the ring-opening and radical capture reactions may be hampered. The ring-closed nortricyclyl radical is thermodynamically favored, for example, over the ring-opened norbornenyl radical.[12] In order to circumvent these limitations and thus obtain an estimate of the rate of the oxygen-transfer reaction, we have employed radical probes that rearrange at increasingly rapid rates. We report here studies of the enzymatic oxidation of nortricyclane, methylcyclopropane, and bicyclo[2.1.0]pentane. The first two substrates are oxidized without rearrangement, but the third is oxidized to a mixture of rearranged and unrearranged products. This is consistent with the fact that the radical derived from the last, highly strained, hydrocarbon rearranges much faster than the cyclopropylmethyl radical.

Materials and Methods

Nortricyclane was a standard sample from the American Petroleum Institute (Carnegie Institute of Technology, Pittsburgh, PA). Methylcyclopropane was purchased from Columbia Organics (Camden, SC); cyclopropylmethanol, cyclobutanol, 3-buten-1-ol, cyclopentanol, cyclopenten-3-one, and norborn-5-en-2-ol from Aldrich Chemical Co. (Milwaukee, WI); and NADPH, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase from Sigma Chemical Co. (St. Louis, MO). 1-Aminobenzotriazole was synthesized as reported in the literature,[13] 2-cyclopenten-1-ol by the procedure of Gemal and Luche,[14] and nortricyclanol by reaction of norbornadiene with 50% sulfuric acid.[15]

Chromatography and Spectroscopy. Analytical gas–liquid chromatography was carried out on a Varian 2100 instrument equipped with flame-ionization detectors. A 6-ft glass column packed with 10% Carbowax 20M on 120/140 mesh Gaschrome Q, a carrier gas flow rate of 30 mL/min, and a column temperature of 90 °C were employed for all the analytical work except that with nortricyclane. Analyses of the metabolism of nortricyclane were carried out at 70 °C on a 6-ft glass column packed with 3% OV-225 on 100/120 mesh Supelcoport. Preparative isolation of metabolites was achieved on the same instrument with 5.5-ft glass columns packed with the same materials by placing a 10:1 splitter prior to the flame-ionization detector. Coupled gas chromatography–mass spectrometry was carried out on a Kratos MS-25 instrument equipped with a 30-m DB-1 capillary column (1.0-μm film thickness). In the case of bicyclo[2.1.0]pentane, the column was held at 40 °C for 15 min after on-column injection of the sample and was then programmed to rise to 80 °C at 4 °C/min; for methylcyclopropane, the same protocol was used except that the column temperature was programmed to rise to 250 °C, and for nortricyclane, the column was programmed to rise to 300 °C at 5 °C/min. Proton NMR spectra were recorded in deuteriated chloroform on a General Electric 500-MHz or custom-built 240-MHz instrument. Chemical shift values are reported in ppm relative to the chloroform peak at 7.24 ppm. Analysis of deuteriated product mixtures was done by NMR. Cytochrome P-450 was quantitated from the dithionite-reduced vs. unreduced CO difference spectra.[16]

Synthesis of Bicyclo[2.1.0]pentane. This compound was prepared as described in the literature by condensation of cyclopentadiene with diethyl azodicarboxylate, reduction of the resulting adduct over palladium/charcoal, hydrolytic removal of the two carbethoxy groups, oxidation over cuprous chloride, and pyrolysis.[17] The spectral properties of the synthetic intermediates and the final product are in full accord with the assigned structures. The deuteriated compound was prepared by the same procedure except that deuterium rather than hydrogen gas was used in the catalytic hydrogenation. Product analyses indicate that approximately 70% of the final product was exo dideuteriated, 20% endo dideuteriated, and no more than 10% undeuteriated. It is likely that the endo-dideuteriated material arises from inversion of the pyrrolytically generated diradical prior to its collapse to give bicyclo[2.1.0]pentane.

Metabolism of Nortricyclane. Preparations of hepatic microsomes from phenobarbital-pretreated rats, obtained as previously reported,[18] contained 4 nmol of P-450/mL, 150 mM KCl, and 1.5 mM DETAPAC in 0.1 M $NaKPO_4$ buffer (pH 7.4). One 80-mL aliquot of the microsomal mixture was preincubated with 1-aminobenzotriazole (2 mM) and NADPH (1 mM) for 30 min at 37 °C, a treatment that reduced the total P-450 concentration to 22% of the initial value as judged by difference spectroscopy. Nortricyclane (5 mM) was then added to the ABT-pretreated incubation and to two other 80-mL aliquots of the liver microsome preparation. NADPH (1 mM) was then added to the ABT-pretreated mixture and to one of the two untreated mixtures, but not to the other, and the three mixtures were incubated for 30 min at 37 °C. The P-450 content of the incubations was measured after this incubation period. The incubations were then extracted with three 50-mL aliquots of diethyl ether, and the extracts from each incubation were combined, dried over sodium sulfate, and concentrated to a volume of 0.5 mL under a stream of argon. The concentrated extracts were then analyzed by gas–liquid chromatography. One peak is found (retention time 3.2 min) that is not present in control incubations. The identity of this metabolite was confirmed by coupled gas chromatography–mass spectrometry. A specific search was made for the isomers of norborn-5-en-2-ol, which have retention times of 1.6 and 1.9 min.

Metabolism of Methylcyclopropane. Methylcyclopropane was bubbled through 75 mL of a preparation of hepatic microsomes from phenobarbital-pretreated rats (3 nmol of P-450/mL) placed in an ice bath. The headspace was then flushed with a 1:4 (v/v) oxygen–nitrogen mixture and the incubation mixture sealed under a slight positive pressure of this gas mixture. The NADPH regenerating system, consisting of 0.3 mM NADP, 3.0 mM glucose-6-phosphate, 0.5 unit/mL glucose-6-phosphate dehydrogenase, and 1 mM $MgCl_2$ in phosphate buffer (pH 7.4), was then added and the mixture incubated for 30 min at 37 °C. Control incubations were carried out similarly in the absence of the NADPH regenerating system or with microsomes that had been pretreated with ABT

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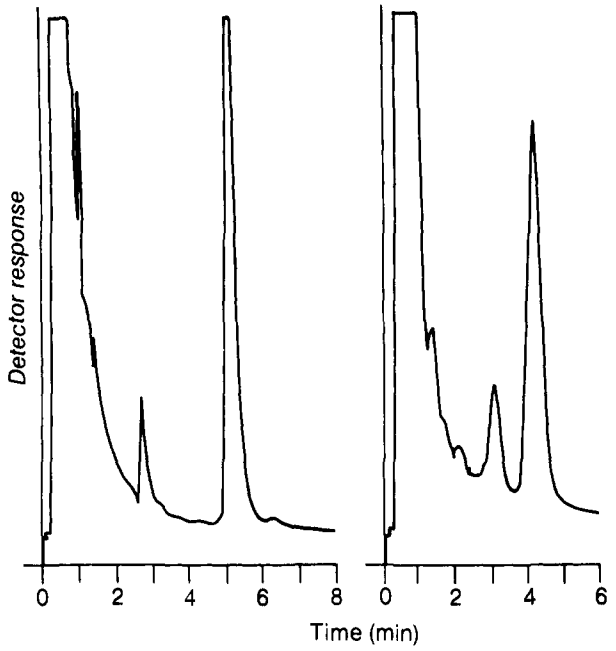


Figure 1. Gas-liquid chromatograms of diethyl ether extracts from incubations of (left) nortricyclane and (right) cyclopropylmethane with liver microsomes from phenobarbital-pretreated rats. The peak at 3 min in the chromatogram on the left coelutes with authentic nortricyclanol. The peak at 3.2 min on the right chromatogram coelutes with cyclopropylmethanol. The large peak at 5.02 min in the left and 4.2 min in the right chromatogram is the internal standard. Details of the incubation and chromatographic procedures are given in the Experimental Section.

as described above. ABT treatment destroyed 90% of the P-450 content of the microsomes as measured by difference spectroscopy. The cytochrome P-450 concentration was measured before and after incubation with methylcyclopropane. In order to obtain quantitative data, 2-heptanone was added as an internal standard prior to extraction of the incubation mixture with diethyl ether. Each incubation was extracted with three 50-mL portions of diethyl ether, and the combined extracts from each incubation were dried over anhydrous sodium sulfate, concentrated to 0.5 mL under a stream of argon, and analyzed by gas-liquid chromatography. Quantitative data were obtained by comparison with a standard curve constructed by adding increasing amounts of authentic material to liver microsomes and working them up as described above. A specific search was made for products with the retention times of authentic cyclobutanol (4.07 min), cyclopropylmethanol (4.01 min), and 3-buten-1-01 (2.86 min).

Metabolism of Bicyclo[2.1.0]pentane. Aliquots (50 mL) of liver microsomes from phenobarbital-pretreated rats (3.3 nmol of P-450/mL) were preincubated with 5 mM bicyclopentane in sealed Erlenmeyer flasks for 5 min at 37 °C. A 30-min enzymatic reaction period was then initiated by injecting the NADPH regenerating system described above. Control incubations without NADPH or the NADPH regenerating system, or with liver microsomes pretreated with 1 mM 1-aminobenzotriazole as described above, were run in the same manner. Aliquots were withdrawn periodically during the incubation, and their cytochrome P-450 content was determined spectroscopically. After 30 min, 2-heptanone was added as an internal standard, the incubations were extracted with three 50-mL portions of diethyl ether, and the combined organic layers were dried over sodium sulfate, concentrated to a final volume of 0.5 mL by careful evaporation in the cold, and analyzed by gas-liquid chromatography.

Results

Metabolism of Nortricyclane and Cyclopropylmethane. Incubation of nortricyclane or methylcyclopropane with microsomal preparations from phenobarbital-pretreated rats, extraction of the products into diethyl ether, and analysis of the extracts by gas-liquid chromatography, yields, in each instance, only one detectable metabolite (Figure 1). Neither metabolite is formed in the absence of NADPH. Furthermore, metabolite formation is severely depressed if the microsomes are preincubated with 1-aminobenzotriazole, a mechanism-based irreversible inhibitor of cytochrome P-450 enzymes.[19,20] The metabolite derived from

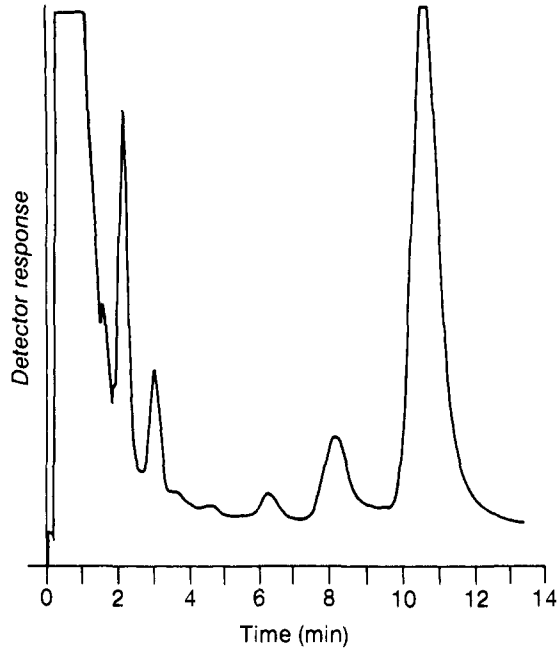
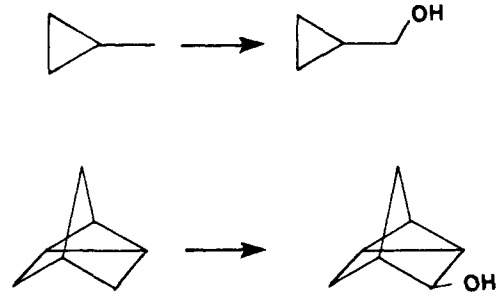


Figure 2. Gas-liquid chromatographic analysis of the diethyl ether extract from an incubation of bicyclo[2.1.0]pentane with hepatic microsomes from phenobarbital-pretreated rats. The peaks at 8.1 and 10.5 min are not observed in the absence of NADPH. 3-Cyclopenten-1-ol coelutes with the material at 8.1 min. The other peaks in the chromatogram, except for that at 6.3 min,[21] are present in control incubations without NADPH and are therefore microsomal constituents.

Scheme I. Metabolism of Nortricyclane and Methylcyclopropane



nortricyclane is identified by its mass spectrum and by coelution with an authentic sample as nortricyclanol. No product is found with the retention time of authentic norborn-5-en-2-ol. The metabolite obtained from cyclopropylmethane, as shown by coelution with an authentic sample, is cyclopropylmethanol. Cyclobutanol and 3-buten-1-ol, two possible rearranged metabolites, were searched for and not found. Quantitative studies indicate that cyclopropylmethanol is formed at a rate of approximately 2 nmol/mL/min, which corresponds to a turnover rate of approximately 0.7 nmol/nmol of P-450/min. No other peak is detected in the chromatogram that is not present in control incubations without cyclopropylmethane. The hydroxylations of nortricyclane and, more surprisingly, methylcyclopropane thus proceed without detectable opening of their cyclopropane ring (Scheme I).

Metabolism of Bicyclo[2.1.0]pentane. Bicyclo[2.1.0]pentane was synthesized by a literature procedure and was incubated with hepatic microsomes from phenobarbital-pretreated rats. Two metabolites, with retention times of 8.1 and 10.5 min, were detected by gas-liquid chromatography in diethyl ether extracts of the incubation mixtures (Figure 2).[21] These products are not

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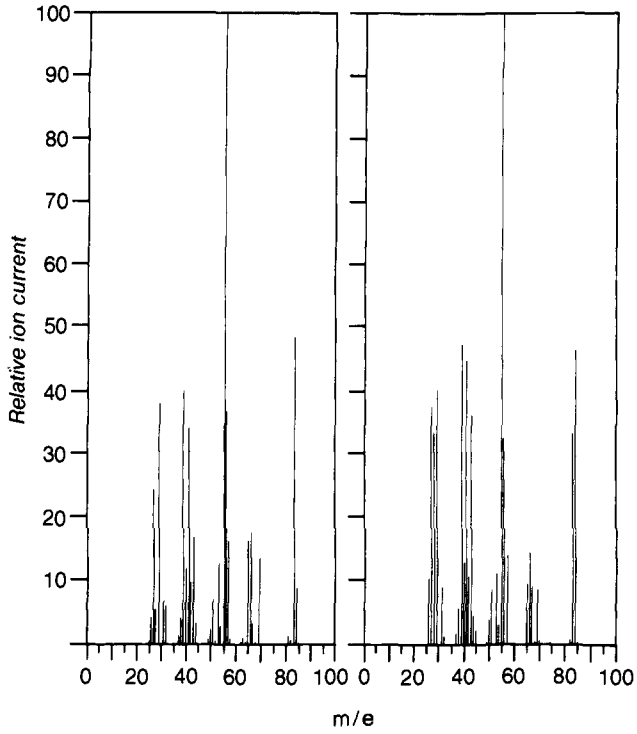

Figure 3. Electron impact mass spectra (70 eV) of the 3-cyclopenten-1-ol (left) and *endo*-2-hydroxybicyclo[2.1.0]pentane (right) metabolites.

observed in the absence of NADPH, and their formation is severely inhibited if the microsomes are preincubated with 1-aminobenzotriazole. Quantitation with respect to an internal standard (2-hexanol) indicates that the product with a retention time of 8.1 min is formed at a rate of approximately 0.4–0.6 nmol/nmol of P-450/min, whereas the product with a retention time of 10.5 min is formed approximately 10 times faster (2.4 nmol/nmol of P-450/min). The ratio of the two products in four independent experiments ranged from 1:6 to 1:10. Collection of the product at 10.5 min and reinjection into the gas chromatograph showed that a trace of the product at 8.1 min is formed in the gas chromatograph but in such low amounts (ratio of 8.1- to 10.5-min peaks after reinjection approximately 1:30) that thermal rearrangement could be neglected in quantitative studies of the metabolically generated products.

The metabolite with a retention time of 8.1 min is shown by mass spectrometric comparison with authentic material to be 3-cyclopenten-1-ol (Figure 3).[22] No trace could be found of the isomeric 2-cyclopenten-1-ol among the metabolites. The product with a retention time of 10.5 min, for which an authentic standard is not available, has been identified by mass and NMR spectroscopies as *endo*-2-hydroxybicyclo[2.1.0]pentane (Figure 4). Its mass spectrum exhibits an M-1 peak at m/z 83 rather than a molecular ion peak, in contrast to 3-cyclopenten-1-ol, which reproducibly exhibits a molecular ion peak at m/z 84 (Figure 3).[22] Isolation of the metabolite by preparative gas–liquid chromatography provided enough material for analysis by 240- and 500-MHz ^{1}H NMR. The proton geminal to the hydroxyl group is found as a five-line multiplet at δ 4.36 in the NMR spectrum (Figure 5). This signal pattern can be attributed to partial overlap of the peaks in a doublet of doublets of doublets with the following coupling constants: $J_{1,2}$ = 8.87, $J_{1,5}$ = 3.74, and $J_{1,3}$ = 4.12 Hz. Decoupling experiments in which the proton at δ 4.36 was irradiated resulted in sharpening or simplification of the signals at δ 2.41 (H_2), 1.87 (H_3), and 1.20 (H_5). The identity of the proton at δ 1.87 as H_3 is confirmed by the fact that its irradiation simplifies the cyclopropane proton signal at 0.66. The upfield location of the peaks at δ 0.66 and 0.79 clearly identifies them as the cyclopropane protons. The peak at δ 1.20 is assigned to H_5 rather than H_2 because the proton cis to the cyclopropane ring and hydroxyl group should be shielded relative to the proton trans to both of these structural elements. The geminal coupling between H_2 and H_5 is 11.6 Hz. In agreement with these assignments, irradiation of H_2 at δ 2.41 simplifies the signals of H_5 at 1.2 and of H_1 at 4.36. Irradiation of the proton at δ 1.45, assigned to H_4, sharpens the peaks due to H_3 (1.87), H_2 (2.4), and H_7 (0.66), as required for the indicated bridge proton. Irradiation of the cyclopropane proton at δ 0.66 collapses the signal at 0.79 to a singlet. The coupling constant between the geminal cyclopropane protons is $J_{6,7}$ = 4.69 Hz. The chemical shifts of the peaks and the coupling constants are essentially identical with those listed by Wiberg and Barth.[23]

Table I. Destruction of Cytochrome P-450

substrate (incubation conditions)	P-450 remaining at indicated time (min), %			
	0	10	20	30
bicyclo[2.1.0]pentane				
complete	100	86	73	63
NADPH	100	99	99	97
nortricyclane				
complete	100			98
NADPH	100			98
methylcyclopropane				
complete	100			70
NADPH	100			100

Oxidation of Dideuteriated Bicyclo[2.1.0]pentane. Deuterium-labeled bicyclo[2.1.0]pentane was synthesized by substituting deuterium for hydrogen gas in the catalytic reduction step. The material obtained was a mixture of the exo-dideuteriated (70%), endo-dideuteriated (20%), and undeuteriated bicyclo[2.1.0]pentane. The endo-deuteriated fraction presumably arises from inversion of the diradical intermediate generated in the thermal ring-closure step. Deuterium substitution does not alter the rate of product formation or the ratio of rearranged to unrearranged metabolites (Figure 6). The deuterium distribution in the metabolites produced by incubation of the labeled substrate with hepatic cytochrome P-450 is only consistent with removal of the endo hydrogen (or deuterium). The integrals expected for each of the protons in the spectrum of *endo*-2-hydroxybicyclo[2.1.0]pentane have been calculated on the assumption that the endo hydrogen or deuterium is exclusively removed (Figure 4). The calculation makes allowance for the heterogeneous distribution of the deuterium label in the substrate. The virtual identity of the predicted and observed values for the integrals clearly shows that the hydroxylation of bicyclo[2.1.0]pentane involves, within the limits of the analytical method, exclusive removal of the endo hydrogen or deuterium. The hydroxylation, which only gives the endo metabolite, thus proceeds with retention of stereochemistry.

Integration of the proton signals in the rearranged metabolite is also only consistent with removal of the endo hydrogen or deuterium. It is furthermore evident from comparison of the calculated integrals for all the possible alternatives with the experimental values that the hydroxyl group is delivered primarily from the endo face (i.e., trans to the deuteriums in the major isomer) (Figure 4). The excellent agreement between the calculated and experimental values indicates that no more than a trace of the reaction could have gone by a path other than endo removal and endo delivery.

Destruction of Cytochrome P-450. The cytochrome P-450 content of the microsomes, determined from the reduced CO vs. the CO difference spectrum, is not decreased by incubation with nortricyclane (Table I). Incubation with cyclopropylmethane and bicyclo[2.1.0]pentane, however, results in time-dependent loss of approximately 30% and 37%, respectively, of the cytochrome P-450 complement of the microsomes (Table I). The destruction

(21) A trace of a third metabolite is formed at approximately 1/100th the rate at which *endo*-2-bicyclo[2.1.0]pentane is formed. This metabolite (retention time 6.3 min) coelutes with authentic cyclopentanol but could not be identified unambiguously due to its low concentration.

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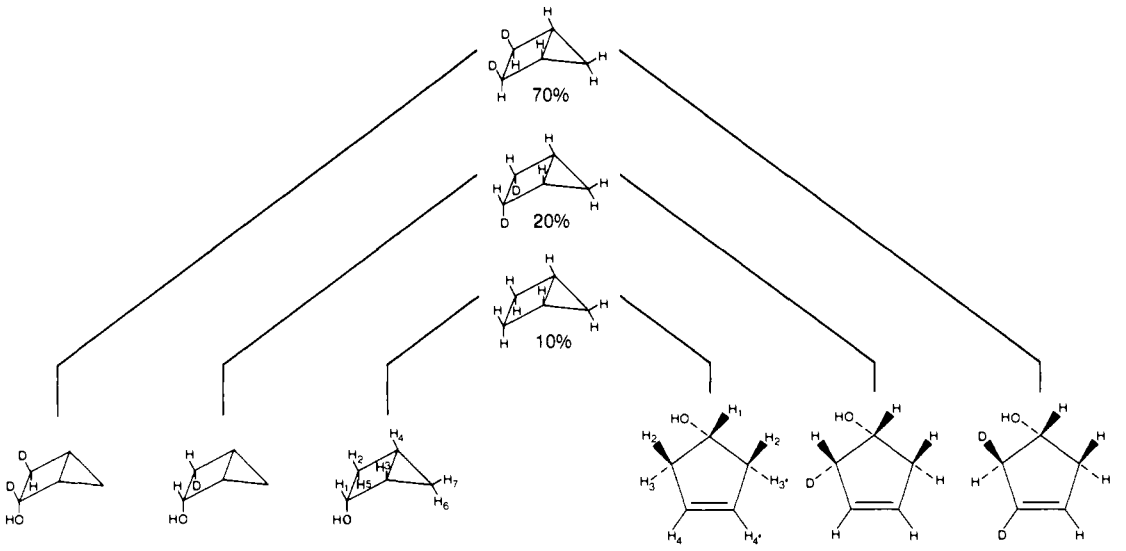

H_1	H_2	H_3	H_4	H_5	H_6	H_7	: Proton :	$H_{4+4'}$	H_1	$H_{2+2'}$	$H_{3+3'}$
4.4	2.4	1.9	1.4	1.2	0.8	0.7	: Chemical shift (ppm) :	5.7	4.5	2.7	2.3
0.3	0.3	1.0	1.0	0.8	1.0	1.0	: Predicted value for integral :	1.3	1.0	1.3	1.8
0.4	0.3	1.0	n.i.	0.9	1.0	0.9	: Actual value for integral :	1.3	1.0	1.3	1.7

Figure 4. Correlation of the NMR signal integrals with the integrals expected for exclusive removal of the endo hydrogen followed by endo and exo delivery of the hydroxyl group, respectively, to give the 2-hydroxybicyclo[2.1.0]pentane and 3-cyclopentenol metabolites. The unrearranged and rearranged products expected from each of the three components of the deuteriated substrate mixture are shown. The integral expected for each proton in the NMR spectrum is given. These integrals take into account the initial deuteriated and undeuteriated substrate mixture. The observed integrals are given in the bottom line.

of cytochrome P-450 by bicyclo[2.1.0]pentane is not detectably altered when the deuterium-substituted substrate is used (Figure 6). Destruction of the monooxygenase is NADPH and substrate dependent, as required for a mechanism-based process.[24] The mechanism of the destructive event remains to be clarified, however, because a "green pigment" analogous to those obtained with a variety of other destructive agents[24] was not detected in the livers of rats treated with bicyclo[2.1.0]pentane.

Discussion

The oxidation of nortricyclane, in agreement with results from other laboratories on norcarane,[8] pericyclocamphanone,[9] and 5,6-methanocholesterol[10] proceeds without detectable rearrangement to nortricyclanol (Scheme I). The interpretation of results with substrates in which the cyclopropylmethyl moiety is integrated into rigid carbon frameworks, however, is complicated by the fact that the cyclopropylmethyl to 3-butenyl radical rearrangement is impaired in such structures. As already noted, the ring-closed nortricyclyl radical is favored thermodynamically over the corresponding ring-opened structure.[12] The nortricyclane rearrangement is thus a radical "clock" of somewhat uncertain calibration but one that almost certainly falls short of the 1×10^8 s^{-1} timed by the cyclopropylmethyl radical. In order to circumvent this limitation, we have investigated the oxidative metabolism of cyclopropylmethane itself, which should give rise to the unencumbered cyclopropylmethyl radical. The finding that this hydrocarbon is oxidized to cyclopropylmethanol without detectably rearranging to 3-buten-1-ol or cyclobutanol (Scheme I) unambiguously establishes that the hydroxylation process is concerted or involves intermediates that recombine at a rate substantially in excess of 1×10^8 s^{-1}. No evidence was found for ring-hydroxylated metabolites, a finding consistent with the fact that hydroxylation of the cyclopropane ring is highly disfavored by the high bond strength of its C–H bonds.

The cyclopropylmethyl radical is the fastest rate-calibrated radical clock currently available in the horlogery of Griller and Ingold.[7] However, radical systems are available that rearrange faster than the cyclopropylmethyl radical but for which the rearrangement rates have not been precisely measured. The bicyclo[2.1.0]pentyl radical is particularly appropriate for such studies because it is structurally related to the cyclopropylmethyl radical, and information is available on the rate of its rearrangement with respect to that of the cyclopropylmethyl radical. The cyclopropylmethyl radical can be prepared at low temperatures and is shown by EPR not to rearrange to the ring-opened radical if the temperature is held below −140 °C.[25] In contrast, the bicyclo[2.1.0]pentyl radical obtained by hydrogen abstraction from the parent hydrocarbon *rearranges completely* to the 3-cyclopentenyl radical by the time the temperature reaches −160 °C.[26] The fact that the bicyclo[2.1.0]pentyl radical rearranges completely at a temperature 20 °C below that at which the cyclopropylmethyl radical does not detectably rearrange implies that the former rearrangement occurs at least 10 times faster than the latter.[27] The bicyclo[2.1.0]pentyl radical thus clocks reactions with rates in excess of 10^9 s^{-1}. The observation that bicyclo[2.1.0]pentane is enzymatically oxidized to a 6:1 mixture of unrearranged and rearranged products (Figure 4) thus (a) confirms that the hydroxylation reaction takes place by a nonconcerted

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(25) Maillard, B.; Forrest, D.; Ingold, K. U. *J. Am. Chem. Soc.* **1976**, *98*, 7024–7026.

(26) Jamieson, C.; Walton, J. C.; Ingold, K. U. *J. Chem. Soc., Perkin Trans. 2* **1980**, 1366–1371.

(27) In the absence of a rate equation for the bicyclo[2.1.0]pentyl radical, a rough estimate can be made of the minimum difference between its rate of rearrangement and that of the cyclopropylmethyl radical. The rates of the cyclopropylmethyl radical rearrangement at −140 and −160 °C are, respectively, 5.4×10^2 and 9.8 s^{-1}.[25] This 55-fold rate difference, and the fact that the bicyclo[2.1.0]pentyl radical is *completely rearranged* at −160 °C whereas the cyclopropylmethyl radical does not detectably rearrange at −140 °C, makes a difference of at least 10 in their rearrangement rates a conservative estimate.

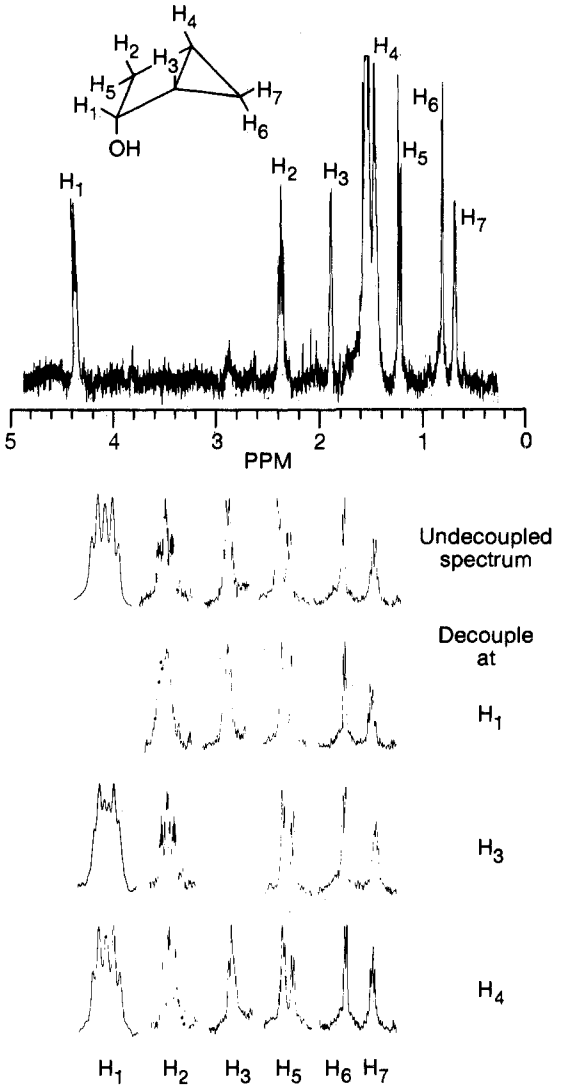


Figure 5. ^1H NMR spectroscopic analysis of metabolically formed *endo*-2-hydroxybicyclo[2.1.0]pentane. The full spectrum is shown at the top. The hydrogen responsible for each of the peaks in the spectrum is identified in the inset structure. The peaks due to all the protons except H_4 are shown in expanded scale below the complete spectrum. The identity of each peak is given at the bottom of the figure. The remaining three spectra show the results of irradiating the proton indicated on the right margin.

process, (b) shows that the process involves a free radical intermediate, and (c) indicates that recombination of the carbon radical with the metal-bound oxygen occurs with a rate similar to that of the bicyclo[2.1.0]pentyl rearrangement (i.e., $k > 10^9$ s^{-1}). This reaction rate, which falls into the range of diffusion-controlled reactions, readily explains why most cytochrome P-450 catalyzed hydroxylations occur without the detectable formation of rearrangement products or the release of carbon radicals from the active site.

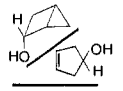
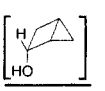
Substrate			% P-450 loss 30 min.
	7.4 ± 0.3	2.8 ± 0.2	50 ± 3
	7.4 ± 0.2	2.5 ± 0.2	45 ± 3

Figure 6. Absence of deuterium isotope effects on the ratio of products and the destruction of microsomal cytochrome P-450. The first column reports the product ratio, the second the amount of bicyclic alcohol (in nmol/min/nmol of P-450), and the third the loss of total P-450 chromophore.

The oxidation of bicyclo[2.1.0]pentane to give the 2-hydroxy product involves removal of the endo hydrogen and delivery of the hydroxyl group to the endo face. The 2-hydroxylation is therefore unexceptional in that it proceeds with retention of stereochemistry. The formation of 3-cyclopenten-1-ol is also initiated by removal of the endo hydrogen and is terminated by delivery of the hydroxyl group to what was the endo face. The two products thus stem from a common initial radical intermediate rather than from the possibly different intermediates produced by removal of the endo and exo hydrogens. The absence of a choice between removal of the exo and endo hydrogens, each leading to a different product, is borne out by the fact that deuteriation does not alter the ratio of the two metabolites or the rate of product formation. The reaction is thus not subject to a primary kinetic isotope effect or to intramolecular "metabolic switching".

The catalysis-dependent destruction of cytochrome P-450 associated with the metabolism of methylcyclopropane and bicyclo[2.1.0]pentane (Table I) suggests that reactive species are produced that destroy the prosthetic heme group, but the absence of a hepatic pigment in at least the case of bicyclo[2.1.0]pentane leaves unanswered the mechanism of the destructive process. The destruction of cytochrome P-450 by these agents joins that mediated, inter alia, by internal acetylenes,[28] allenes,[29] and benzothiadiazoles,[30] all of which destroy the chromophore of the enzyme without detectably forming N-alkylated porphyrins.[24]

In sum, the present results add to the evidence for a nonconcerted hydroxylation mechanism for cytochrome P-450 and specifically implicate a radical pair intermediate that collapses at a rate in excess of 10^9 s^{-1}. The information available on enzymatic hydrocarbon hydroxylations thus uniformly supports the following mechanism (the ferryl heme complex is denoted by [FeO]):

$$[FeO]^{3+} + C\text{-}H \rightarrow [FeOH]^{3+} + C^{\bullet} \rightarrow [Fe]^{3+} + C\text{-}OH$$

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