Cytochrome P450 Hydroxylation of Hydrocarbons: Variation in the Rate of Oxygen Rebound Using Cyclopropyl Radical Clocks Including Two New Ultrafast Probes†

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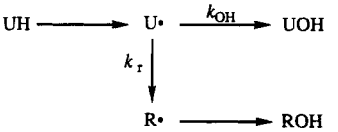
ABSTRACT: The oxidation of eight methyl-substituted and three alkyl-substituted cyclopropanes by rat liver microsomal cytochrome P450 and pure reconstituted rabbit P450 2B4 was studied. Alkane hydroxylation catalyzed by P450 is generally believed to proceed by hydrogen abstraction followed by reaction of the carbon-centered radical with an iron-bound hydroxyl radical, a process called oxygen rebound. Hydrogen abstraction from methylcyclopropanes generates cyclopropylcarbinyl radicals whose solution rate constants for ring opening are known [Bowry, V. W., *et al.* (1991) *J. Am. Chem. Soc. 113,* 5687–5698]. Rearranged products were only observed with the five substrates which, upon hydrogen abstraction, would generate a cyclopropylcarbinyl radical that undergoes ring opening with a rate constant $\geq 2.0 \times 10^9$ s^{-1} in solution. Values of the rate constants for oxygen rebound (k_{OH}) were calculated by determining the ratio of unrearranged products (cyclopropylmethanols) to rearranged products (alkenols). For each substrate this ratio was generally about the same for the oxidations catalyzed by microsomal P450 and by P450 2B4. It is concluded that all of the substrates are oxidized via an intermediate cyclopropylcarbinyl radical. Two ultrafast probes, *trans*-1-methyl-2-phenylcyclopropane and 1,1-diphenyl-2-methylcyclopropane, gave alcohol product ratios which yielded unreasonably high values for k_{OH}, *viz.*, *ca.* 1.5×10^{12} and *ca.* 7×10^{12} s^{-1}, respectively. It would appear to be likely that, with these two probes, the enzyme "holds" the phenyl group(s) in the intermediate cyclopropylcarbinyl radicals in a conformation which reduces the ring-opening rates relative to their rates in solution (in the least favorable conformation the rate of ring opening and hence the calculated k_{OH} values would be reduced by *ca.* 3 orders of magnitude). Three of the substrates examined are unlikely to have the ring-opening rates of their cyclopropylcarbinyl radicals significantly influenced by the enzyme. These probes yield $k_{OH} \sim 2.4 \times 10^{11}$ s^{-1} for oxygen rebound to a primary alkyl radical and about an order of magnitude slower for rebound to a secondary alkyl radical.

The catalytic cycle for the cytochrome P450 monooxygenases has now been described in considerable detail (Ortiz de Montellano, 1987, 1989; Guengerich & Macdonald, 1990; Poulos & Raag, 1992). Evidence exists for the intermediacy of a common oxo-iron species (Fe^{IV}=O) that can account for the variety of oxidative processes catalyzed by these enzymes (Ortiz de Montellano, 1986; Akhtar & Wright, 1991) including alkane hydroxylation, heteroatom dealkylation and oxidation, alkene epoxidation, and dehydrogenation (Guengerich, 1989, 1990). There is also a reactive Fe^{III}—OOH species that is involved in carbon–carbon bond cleavage (Akhtar *et al.*, 1982; Jefcoate, 1986; Corina *et al.*, 1991; Roberts *et al.*, 1991; Vaz *et al.*, 1991). It is now widely accepted that during the P450-catalyzed hydroxylation of alkanes an intermediate carbon-centered radical (Groves, 1985; White, 1991) combines with an iron-bound hydroxyl radical in a process termed "oxygen rebound" (Groves *et al.*, 1978). Our laboratory has recently calibrated a series of cyclopropylcarbinyl radical rearrangements utilizing the nitroxide radical trapping technique (Bowry *et al.*, 1991). The parent alkylcyclopropanes (UH in Scheme I) were then used as "radical clock" substrates (Griller & Ingold, 1980; Bowry *et al.*, 1989) to investigate the hydroxylation reaction catalyzed by cytochrome P450 (Bowry & Ingold, 1991). If, as suspected, alkane hydroxylation is initiated by hydrogen abstraction, then the carbon radical so produced (an unrearranged cyclopropylcarbinyl radical, U• in Scheme I) may be hydroxylated directly or it may undergo ring cleavage to give a rearranged radical R•, which can also be hydroxylated. An analysis of the product alcohol ratio, UOH/ROH, provides a measure of the competition between the rate of oxygen rebound and the previously calibrated rate of rearrangement. Ortiz de Montellano and Stearns have reported that methylcyclopropane gave only the unrearranged alcohol, cyclopropylmethanol. Since the rate constant for ring opening, k_r, of the cyclopropylcarbinyl radical is 1.2×10^8 s^{-1} at 37 °C, they reported the rate constant for oxygen rebound, k_{OH}, to be $>10^8$ s^{-1} (Ortiz de Montellano & Stearns, 1987). They also demonstrated that phenobarbital-induced rat liver microsomes oxidized bicyclo[2.1.0]pentane to give both rearranged and unrearranged product alcohols. From these results they were able to estimate the rate constant for oxygen rebound to be "in excess of 10^9 s^{-1}" (Ortiz de Montellano & Stearns, 1987). Our recent work with bicyclo[2.1.0]pentane has determined that $k_{OH} = 2.2 \times 10^{10}$ s^{-1} for this substrate (Bowry & Ingold, 1991). We report herein our

Scheme I



$k_r \times \text{UOH/ROH} = k_{OH}$

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reinvestigation of the oxidation of these and other alkylated cyclopropanes catalyzed by phenobarbital-induced rat liver microsomal P450 as well as their oxidation catalyzed by purified rabbit P450 2B4. We have also studied the oxidation of two of Professor Newcomb's "ultrafast" radical clock substrates; *trans*-1-methyl-2-phenylcyclopropane (Newcomb & Manek, 1990; Fu *et al.*, 1991) and 1,1-diphenyl-2-methylcyclopropane (Newcomb *et al.*, 1992), by these two forms of P450.

EXPERIMENTAL PROCEDURES

Instrumentation. Product analyses were performed on a FID-equipped HP5890 Series II gas chromatograph with a cross-linked methyl silicone column (HP-1, 50 m × 0.22 mm i.d. or 10 m × 0.20 mm i.d.). The carrier gas was helium, and the temperature program was 40 °C for 5 min raised at 10 °C/min to 180 °C for the methyl-substituted cyclopropanes on the 50-m column. The phenyl-containing substrates were chromatographed on the 10-m column with the temperature program 90–110 °C for 4 min raised at 10 °C/min to 220 °C. The injection temperature for both methods was 220 °C. Mass spectral analyses were done using a Hewlett-Packard HP 5890 GC instrument and a cross-linked methyl silicone column (HP 1, 12 m × 0.2 mm i.d.) interfaced with an HP 5970 (EI, 70 eV) mass selective detector.

Materials and Methods. Some of the substrate cyclopropanes and product alcohols were commercially available and others were synthesized. Sources and methods of synthesis have been previously described (Bowry & Ingold, 1991). Epoxides were synthesized following a general procedure (Pasto & Cumbo 1965) which involved a 3-chloroperbenzoic acid epoxidation of an ice-cold solution of the alkene. The clock substrates *trans*-1-methyl-2-phenylcyclopropane and 1,1-diphenyl-2-methylcyclopropane were kindly provided by Cathy Johnson and Prof. Martin Newcomb, Department of Chemistry, Wayne State University, Detroit, MI. The ring-closed oxidation product of isopropylcyclopropane, 2-cyclopropyl-2-propanol, was prepared by addition of MeMgBr to cyclopropyl methyl ketone, both purchased from Aldrich. The synthesis of *trans*-1-benzyl-2-phenylcyclopropane has been recently described (Hollis *et al.*, 1992). All products gave appropriate mass spectral and NMR analyses.

Microsomes were prepared from phenobarbital-treated rats (80 mg/kg of body weight) following literature methods (Ortiz de Montellano *et al.*, 1981) and were stored at −80 °C in small aliquots to avoid multiple freeze/thaw cycles. The concentration of P450 in the microsomes was determined following the method of Estabrook (Estabrook *et al.*, 1972). As prepared, the microsomes represented 1.2–1.5 g of liver/mL of final suspension and generally contained 20–30 mmol of P450/mL.

Reaction Procedures. Incubations with the microsomal P450 were designed so that yields of the product alcohols could be determined over time for up to 60 min rather than making all of the measurements at a single time (typically 30 min) as has been the usual practice (Ortiz de Montellano & Stearns, 1987; Bowry & Ingold, 1991). For a typical experiment with a dimethyl-substituted cyclopropane the microsomes were diluted 4× with buffer (50 mM sodium phosphate, 1 mM desferrioxamine, 0.075 M KCl, pH 7.4) to give P450 concentrations of approximately 5–7 nmol/mL. (The microsomes were diluted 10× for *trans*-1-phenyl-2-methylcyclopropane and 3× for 1,1-diphenyl-2-methylcyclopropane.) Two 20-mL glass scintillation vials were used for a single run, each containing 8.5 mL of the diluted microsomes. To each of these vials was added 10 μL of the liquid alkane. The mixture was allowed to sit on ice for at least 10 min with occasional swirling by hand and was then placed in a shaking water bath thermostated at 37 °C for 2 min. Reactions were initiated by the addition of NADPH (10 mg of the tetrasodium salt) in 0.5 mL of buffer to yield a final concentration of 1.2 mM. At the appropriate time points, 2 mL of the mixture was removed and placed in a test tube containing 2 mL of ice-cold buffer to stop the reaction. After all samples had been collected, 200 μL of 0.5 M sodium dodecyl sulfate was added to each tube, which was then vortexed for 30 s. An internal standard was added—300 nmol of cyclohexanol in 40 μL of EtOH for the more volatile substrates or a similar amount of cyclooctanol for the phenyl-containing substrates. The mixture was extracted with distilled CH_2Cl_2, 3 × 2 mL. The vortexed extracts were centrifuged for 5 min at 3000*g* to separate the phases. Phase separation could be made more distinct by adding 100 mg of NaCl to each tube. For reactions where the microsomes had been diluted 10× it was helpful to add 100–150 μL of the concentrated microsomes to the extraction mixture to achieve good phase separation. The organics were combined, dried over anhydrous $MgSO_4$, and then evaporated under a gentle stream of nitrogen to 250 μL or less and analyzed by GC. Yields of the alcohol products were determined from known GC response factors calculated from standard injections.

Reconstituted P450 2B4. Pure rabbit P450 2B4 and NADPH cytochrome P450 reductase were generous gifts from Dr. M. J. Coon, Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor, MI. The enzymes were reconstituted according to previous methods (White *et al.*, 1980) using dilaurylphosphatidylcholine (DLPC) at a final concentration of 100 μg/mL and a ratio of P450 to reductase of 1:2. Each sample for analysis also contained 1000 units of catalase and 60 units of SOD (both from Sigma) to combat adventitious production of H_2O_2 and O_2^- by the cytochrome/reductase couple. Only one incubation time of 20 min was used for each substrate.

RESULTS

The product profiles obtained from microsomal oxidation of five polymethylated cyclopropanes and bicyclo[2.1.0]-pentane are illustrated in Figure 1. One can clearly see the production of the unrearranged alcohols for all substrates. With the microsomes diluted 4× the maximum yield of alcohol was achieved within approximately 15 min. Figure 1 also shows the product profiles obtained from the oxidation of *trans*-1-methyl-2-phenylcyclopropane (**10H**), for which microsomes were diluted 10×, and 1,1-diphenyl-2-methylcyclopropane (**11H**), for which microsomes were diluted 3×. Except for *trans*-2-phenylcyclopropylmethanol (**10OH**), all of the unrearranged alcohols were stable to the reaction medium as shown by their generally flat profiles from *ca.* 15 min up to 60 min. However, **10OH** appears to be further oxidized by the microsomes since its disappearance from the reaction mixture was dependent on both the concentration of the microsomes and the presence of NADPH (data not shown). When the microsomes were diluted 10×, as they were for the data illustrated in Figure 1, the disappearance of **10OH** apparently occurred with a concomitant loss of the rearranged alcohol, 1-phenyl-3-buten-1-ol (**10rOH**), since the ratio **10OH**/**10rOH** remained relatively constant. Microsomes also oxidized **10H** and **11H** to give significant yields of phenols (data not shown); approximately 300 nmol for **10H** and 200 nmol for **11H** after a 20-min incubation under the same

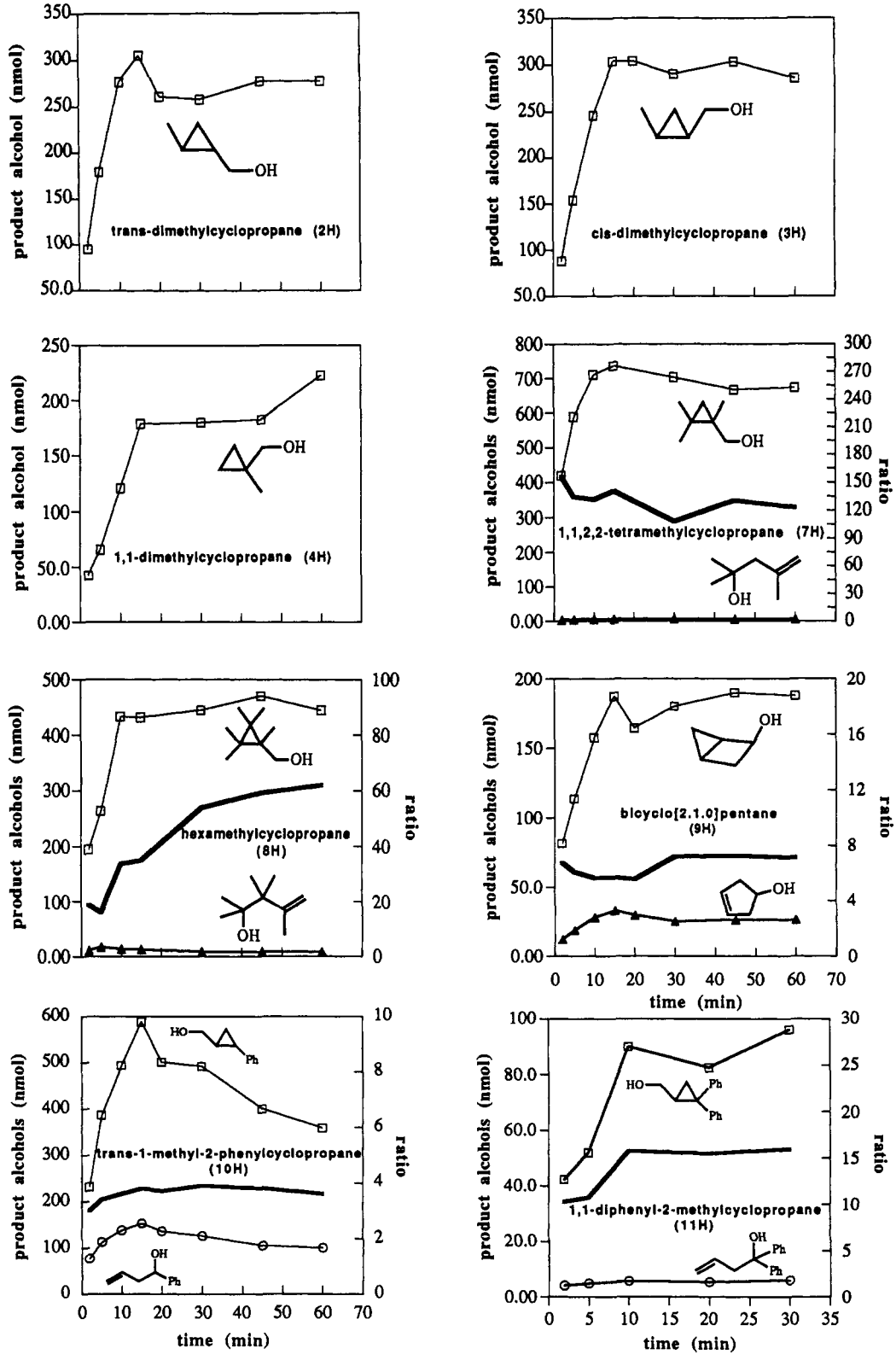


FIGURE 1: Alcohol product profiles for the rat liver microsomal oxidations of various substituted cyclopropanes. The bold lines, when present, are the ratios of the two product alcohols UOH/ROH.

conditions as Figure 1. The yields of unrearranged alcohols were higher for the tetra- and hexamethylcyclopropanes and for **10H** than for those substrates with only two methyl substituents and for bicyclo[2.1.0]pentane. The recovered yield of the rearranged product alcohol, **8rOH**, from the microsomal oxidation of hexamethylcyclopropane (**8H**) also diminished over the 60-min incubation.

One of us originally reported (Bowry & Ingold, 1991) that small amounts of the two possible rearranged alcohols, 4-penten-2-ol [**2r(s)OH**] and 3-methyl-3-buten-1-ol [**2r(p)-OH**], were detected after the microsomal oxidation of both *trans*- and *cis*-dimethylcyclopropane (**2H** and **3H**, respectively). *We have now discovered that this is not the case* by using an improved extraction method and a much longer GC column which allowed a better separation of low-abundance products from solvent impurities and materials extracted in extremely small amounts from the microsomes themselves. Despite several attempts, we have failed to detect any **2r(s)-**

Scheme II

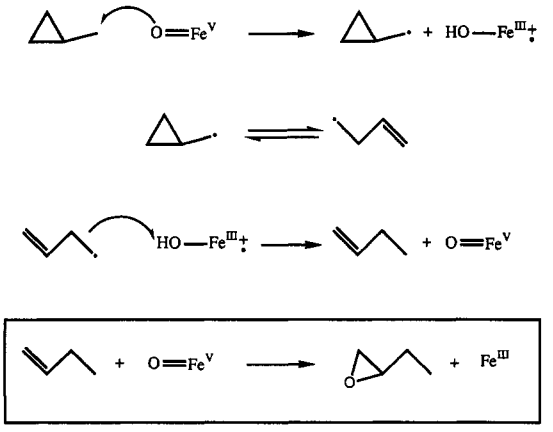


Table I: Relative Product Yields and Lower Limits on the Oxygen-Rebound Rate Constants (k_{OH}) for P450-Catalyzed Hydroxylation of Some Alkyl-Substituted Cyclopropanes That Yield Slow Radical Clocks (UH → UOH + ROH)

U·	$k^{37°}$ (10^8 s^{-1})[a]	R·	Products [UOH]:[ROH] P-450 2B4	Products [UOH]:[ROH] Microsomes	Microsomal[b] "k_{OH}" (s^{-1})
1·	1.2	1r·		> 100:1	**> 1.2 x 10^{10}**
2·	1.6	2r(s)·	>100:1	> 100:1	**> 1.6 x 10^{10}**
	1.8	2r(p)·	> 120:1	> 100:1	**> 1.8 x 10^{10}**
3·	8.0	2r(s)·	> 100:1	> 100:1	**> 8.0 x 10^{10}**
	2.3	2r(p)·	> 100:1	> 100:1	**> 2.3 x 10^{10}**
4·	0.8	4r·	> 180:1	> 180:1	**> 1.4 x 10^{10}**
5·	0.88	5r·		> 120:1	> 1.1 x 10^{10}
6·	3.6[c]	6r·		—	—

[a] Rate constants have been taken from Bowry *et al.* (1991) unless otherwise noted. [b] Lower limits on k_{OH} considered to be fairly reliable (see text) are given in boldface. [c] Hollis *et al.* (1992).

OH or **2r(p)**OH in oxidations of **2**H and **3**H using several different batches of microsomes. In case these two rearranged alcohols were not formed from the dimethylcyclopropanes because the compounds reacted in the manner depicted (for methylcyclopropane) in Scheme II, the extracts were also analyzed for epoxides. However, neither 1,2-epoxy-3-methylbutane nor 1,2-epoxypentane could be detected as a product of these reactions either by inspection of GC traces or by cochromatography with standards.

trans-1-Phenyl-2-methylcyclopropane (**10**H) was oxidized much more rapidly than any of the polymethylcyclopropanes, for which reason the microsomes were diluted 10× so that the product curves could be more easily observed. By way of contrast, 1,1-diphenyl-2-methylcyclopropane (**11**H) was a relatively poor substrate and the products were only identified when more concentrated microsomes were used (dilution 3×).

Tables I and II summarize the previously obtained rate constants for ring opening of the cyclopropylcarbinyl radicals (Bowry *et al.*, 1991; Newcomb *et al.*, 1992) and the product

Table II: Relative Product Yields and Oxygen-Rebound Rate Constants (k_{OH}) for P450-Catalyzed Hydroxylation of Some Alkyl-Substituted Cyclopropanes That Yield Fast Radical Clocks (UH → UOH + ROH)

U·	$k^{37°}$ (10^8 s^{-1})[a]	R·	Products [UOH]:[ROH] P-450 2B4	Products [UOH]:[ROH] Microsomes	Microsomal[b] "k_{OH}" (s^{-1})
7·	20	7r(t)·	106:1	124:1	**2.5 x 10^{11}**
	2.1	7r(p)·	> 200:1	> 200:1	**> 4.2 x 10^{10}**
8·	47	8r·	50:1	48:1	**2.3 x 10^{11}**
9·	21[c]	9r·	11:1[d] 17:1	6.5:1 (7:1)[e]	**1.4 x 10^{10}**
10·	~4000[f,g]	10r·	3.0:1	3.8:1	~2 x 10^9 to ~1.5 x 10^{12}[h]
11·	~5000[g]	11r·	3.5:1	14.5:1	~7 x 10^9 to ~7 x 10^{12}[h]

[a] Rate constants have been taken from Bowry *et al.* (1991) unless otherwise noted. [b] Values of k_{OH} considered to be fairly reliable (see text) are given in boldface. [c] Bowry *et al.* (1991); Newcomb *et al.* (1991). [d] Two separate analyses with identically prepared reconstituted enzymes. [e] Ortiz de Montellano and Stearns (1987). [f] Newcomb and Manek (1990). [g] Newcomb *et al.* (1992). [h] See text for an explanation for this wide range of k_{OH} values.

ratios or limits thereon obtained from the present microsomal P450 and reconstituted P450 2B4 oxidations. The rate constants (or limits thereon) calculated for oxygen rebound are also given in these tables.

DISCUSSION

For all of the clock substrates utilized in this work the unrearranged alcohols UOH were the most abundant products. Unless a cyclopropylcarbinyl radical has a rate constant for ring opening *ca.* ≥20 × 10^8 s^{-1} in solution, the *rearranged* product alcohol either is not formed or is formed in amounts below the current limits of detection. In experiments with the dimethylcyclopropanes the limit of detection for ROH was approximately 1–2% of UOH, or about 6 nmol of the product in the 2-mL aliquot that was extracted.

Rabbit P450 2B4 is an orthologous form of rat P450 2B2 with which it shares extensive sequence homology (Black & Coon, 1986; Gonzalez, 1989, 1990). Reassuringly, the product ratios determined with the reconstituted system are generally close to those found with the microsomes. This supports the assumption that the most abundant form of P450 in phenobarbital-induced rat microsomes, P450 2B2, is largely responsible for the observed oxidation products and their relative yields.

Bicyclo[2.1.0]pentane (**9**H) is one of the poorest substrates for P450 (see Figure 1); nevertheless, the ratio **9**OH/**9r**OH determined from microsomal oxidation of the bicyclopentane agreed very well with that reported originally, *viz.*, 7:1 (Ortiz de Montellano & Stearns, 1987), indeed better than with the ratio reported subsequently, *viz.*, 10.5:1 (Bowry & Ingold, 1991). However, the **9**OH/**9r**OH ratios determined using

the P450 2B4 reconstituted system were, for reasons unknown, approximately twice as large and were variable.

The limit for detection of **4r**OH, the putative rearrangement product of oxidation of 1,1-dimethylcyclopropane (**4**H), was better than for the other methylcyclopropanes (see Table I) because the GC chromatogram was particularly free of interference from impurities around the **4r**OH retention time. Nevertheless, **4r**OH could not be detected.

The tertiary alcohol **7r(t)**OH was found as a rearranged product from the oxidation of 1,1,2,2-tetramethylcyclopropane (**7**H), but the rearranged primary alcohol **7r(p)**OH could not be detected.

The tertiary alcohol **8r**OH, which is the sole possible rearranged product from the oxidation of hexamethylcyclopropane (**8**H), was formed but appears to be unstable in the reaction medium, slowly disappearing over the course of the incubation. Dehydration of this alcohol to form 2,3,3,4-tetramethyl-1,4-pentadiene seems to be probable, but the diene could not be detected in the extracts by selected ion GC/MS [as previously noted (Bowry & Ingold, 1991)]. However, the *ca.* 10-fold loss of sensitivity which occurs on changing from a gas chromatograph equipped with a flame ionization detector to a GC/MS system would almost certainly have precluded observation of the diene in view of the low yields of **8r**OH. Because of the slow disappearance of **8r**OH, the ratio **8**OH/**8r**OH increases from *ca.* 20 at the start of the reaction to *ca.* 60 after 60 min.

In the calculation of the k_{OH} values given in Tables I and II, it was at first *assumed* that the enzyme did not place any constraints on the radical rearrangements and that, should a cyclopropylcarbinyl radical be formed, it would ring-open with the same rate constant as in free solution. This assumption is quite likely to be valid *unless* the cyclopropylcarbinyl radical possesses a structure which causes it to be generated and "locked" within the enzyme's active-site "pocket" in a conformation which is *unfavorable* to ring opening. That is, the most favorable conformations for ring opening can readily be achieved in solution since bond rotations are not restricted by the surrounding medium. For this reason, k_r (Scheme I) probably has its maximum value in solution. By way of contrast, in a conformationally constrained enzymic environment the ring-opening rate "constant" might be reduced by enforced unfavorable stereoelectronic factors. This potential problem in calculating lower limits and actual values for k_{OH} (Tables I and II) can, we believe, safely be ignored for the simple, methyl-substituted cyclopropane substrates, **1**H–**4**H, **7**H, and **8**H, for the reason that the only bond rotation of concern is that about the cyclopropyl–$CH_2^{\bullet}$ bond. It would be extremely difficult for external forces to retard or prevent this rotation, which puts the radical into the stereoelectronically favored conformation for ring opening [in which there is maximum overlap between the singly occupied molecular orbital (SOMO) and the cyclopropane bond which will be broken]. For the bicyclopentyl radical, **9**$^{\bullet}$, there is no question of bond rotation and hence k_r should also be more-or-less unaffected by the local environment. Thus, for substrates **1**H–**4**H and **7**H–**9**H we believe the lower limit or actual values listed for k_{OH} are probably reliable. These values are given in boldface in Tables I and II.

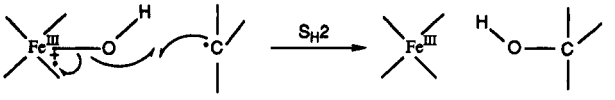
The potential for enzymically induced conformational restrictions on the cyclopropylcarbinyl radical which could cause a reduction in the rate of ring opening is present with radicals **5**$^{\bullet}$, **6**$^{\bullet}$, **10**$^{\bullet}$, and **11**$^{\bullet}$. Since no alcoholic products at all could be detected after attempted microsomal and P450 2B4 oxidation of **6**H, this substrate need concern us no longer. The ring opening of the tertiary alkyl radical from isopropylcyclopropane (**5**$^{\bullet}$) is relatively slow in solution ($k_r = 0.88 \times 10^8$ s^{-1}, Table I), and since only the unrearranged tertiary alcohol, **5**OH, could be detected, this probe also need no longer concern us.

With the phenyl-substituted cyclopropylcarbinyl radicals **10**$^{\bullet}$ and **11**$^{\bullet}$, there is a real possibility that the enzyme will enforce conformations in which the π-electron system of the phenyl ring(s) cannot overlap fully with the bond which will break in the cyclopropane ring. This possibility has been considered by Liu *et al.* (1993) in their study of the oxidations of **10**H and **11**H by methane monooxygenase from *Methylococcus capsulatus* (Bath). Using Marcus theory, these workers have estimated that, if the phenyl π-system in **10**$^{\bullet}$ were orthogonal (90°) to the C_2–C_1 bond in the ring, then the 25 °C rate constant for ring scission would decrease from its value of 3×10^{11} s^{-1} (*i.e.*, 4×10^{11} s^{-1} at 37 °C) in free solution to *ca.* 4×10^8 s^{-1} (*i.e.*, 5×10^8 s^{-1} at 37 °C). Thus, from the measured ratio of unrearranged to rearranged alcohols, [**10**OH]/[**10r**OH] = 3.8, the calculated value of k_{OH} (at 37 °C) could range from $3.8 \times 4 \times 10^{11} \approx 1.5 \times 10^{12}$ s^{-1} (no enzymic restriction on conformation or a favorable restriction) to $3.8 \times 5 \times 10^8 \approx 2 \times 10^9$ s^{-1} (restriction to the most unfavorable conformation). Similarly, if we assume that, in an unfavorable conformation, the rate constant for ring opening of **11** will also be decreased by *ca.* 3 orders of magnitude from its value in free solution, then we can estimate that with **11**H as the probe k_{OH} lies between 7×10^{12} and 7×10^9 s^{-1}.

Both the probable lower and the upper limits on k_{OH} calculated from the relative yields of unrearranged/rearranged alcohols produced from **10**H and from **11**H have been included in Table II. The upper limits would imply that oxygen rebound occurred on the time scale of a few molecular vibrations. Such extremely fast oxygen rebound relates to a mechanistic problem which has been noted previously (Ortiz de Montellano, 1987); *viz.*, P450-catalyzed hydroxylations which occur by rebound may "masquerade" as concerted, oxene-insertion processes when the carbon-centered radical and the iron-coordinated hydroxyl radical "collapse" to the alcohol product before the radical can invert, reorient itself, or, in the present case, rearrange. In previously identified examples of this phenomenon, specific P450 isozymes provide the appropriate architecture for substrate orientation so that oxygen rebound follows hydrogen abstraction without the formation of stereochemical or regiochemical isomeric products, as has been nicely described for $P450_{cam}$ (Sligar & Murray, 1986; Atkins & Sligar, 1988; Stayton & Sligar, 1990) and for the sterol metabolizing enzymes (Jefcoate, 1986). In the case of **10**$^{\bullet}$ and **11**$^{\bullet}$ we *cannot rule out a very fast oxygen rebound, but we consider it to be more probable that these two ultrafast radical clocks have "run slow" in the enzyme and that the "true" values for k_{OH} are lower by 1 or 2 orders of magnitude than the upper limits.* [We note that "high" k_{OH} values could also arise if ring opening placed the new radical center in a position remote from the iron–hydroxyl moiety, *provided* the rearranged radical was immobilized by the enzyme *and* that ring opening was reversible. For the two ultrafast probes this source of error can be ruled out because the rearrangement equilibrium lies much too far on the side of the ring-opened radical; e.g., Liu *et al.* (1993) have estimated that K(**10**$^{\bullet}$ ⇄ **10r**$^{\bullet}$) ~ 2×10^{13}, corresponding to k(**10r**$^{\bullet}$ → **10**$^{\bullet}$) ~ 0.01 s^{-1}!]

The simple radical clock approach to "timing" the rate of oxygen rebound in P450-catalyzed alkane hydroxylations is clearly inappropriate if there is *any* likelihood that the clock

could be made to run slow by the enzyme. Fortunately, the clocks **1•–4•** and **7•–9•** are very unlikely to be significantly influenced by the enzyme. For methylcyclopropane (**1H**) and the three dimethylcyclopropanes (**2H–4H**) we have only lower limits for k_{OH}, ranging from 1.2×10^{10} to 8.0×10^{10} s^{-1} (Table I). However, we do have what we believe to be three fairly reliable calculated values for k_{OH}, *viz.*, $\sim 2.4 \times 10^{11}$ s^{-1} for the tetra- and hexamethylcyclopropanes (**7H** and **8H**, respectively) and $\sim 1.4 \times 10^{10}$ s^{-1} for the bicyclopentane (**9H**) (Table II). We tentatively attribute the slower oxygen rebound found with **9H** to the fact that **9•** is a *secondary* alkyl radical, whereas **7•** and **8•** are primary alkyl radicals. Secondary alkyl radicals are generally less reactive than primary alkyls, and we can see no reason why this should not also hold true for the oxygen-rebound step in the P450 catalytic cycle since this step probably involves a bimolecular homolytic substitution, S_H2, at oxygen of carbon for iron, *i.e.*,



SUMMARY

The rat microsomal P450 and rabbit P450 2B4 catalyzed oxidations of the bicyclopentane (**9H**) and four methyl-substituted cyclopropanes (**7H**, **8H**, **10H**, and **11H**) have yielded, in each case, a mixture of the unrearranged, ring-closed, and the rearranged, ring-opened, alcohol products. Furthermore, for most of these substrates the two P450 systems have yielded rather similar ratios of the unrearranged alcohol to rearranged alcohol. We conclude that P450 oxidizes these five hydrocarbons to alcohols via an intermediate carbon-centered radical, *i.e.*, via the oxygen-rebound mechanism (Groves *et al.*, 1978). The same mechanism almost certainly holds true for methylcyclopropane (**1H**) and for three dimethylcyclopropanes (**2H–4H**). However, in the present work we found that two of the 1,2-dimethylcyclopropanes (**2H** and **3H**) did not give rearranged alcohols as we had originally reported (Bowry & Ingold, 1991).

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