A Radical Clock Investigation of Microsomal Cytochrome P-450 Hydroxylation of Hydrocarbons. Rate of Oxygen Rebound[1]

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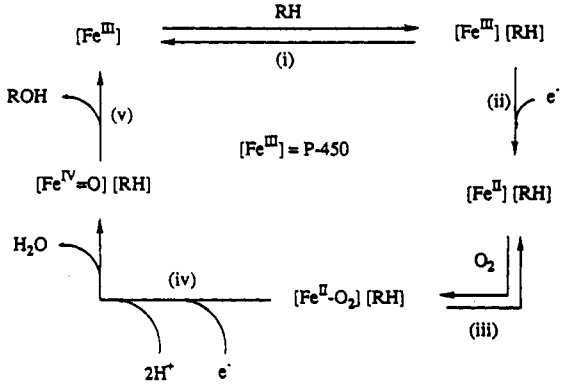
Abstract: A number of alkyl-substituted cyclopropanes for which the rates of ring opening of the corresponding cyclopropylcarbinyl radicals have been determined (see preceding paper in this issue) have been used as substrates for hydroxylation by phenobarbital-induced, rat liver microsomal cytochrome P-450 at 37 °C. Three of these compounds gave both ring-closed and ring-opened alcohols, thus allowing the rate constant, k_{OH}, for "oxygen rebound" onto the corresponding carbon-centered radicals to be determined. In particular, both *trans*- (**1bH**) and *cis*- (**1cH**) 1,2-dimethylcyclopropane gave 4-penten-2-ol (**2bOH**) and 2-methyl-3-buten-1-ol (**3bOH**) together with the corresponding *trans*- (**1bOH**) or *cis*- (**1cOH**) 2-methylcyclopropanemethanols. Of much greater importance, for both **1bH** and **1cH** the ratios of the yields of the secondary-to-primary ring-opened alcohols, i.e., [**2bOH**]/[**3bOH**], were the same, within experimental error as the ratio of the rates of ring opening of the corresponding *trans*- (**1b**•) and *cis*- (**1c**•) methylcyclopropylmethyl radicals in solution at 37 °C. This indicates that when **1b**• and **1c**• are formed from their parent hydrocarbons by H-atom abstraction in the hydrophobic pocket of cytochrome P-450 they are not detectably constrained in their subsequent reactions by their unusual environment. From the ratio of the yields of the unrearranged alcohol to each of the rearranged alcohols we calculate k_{OH} values of 1.5 and 1.6 × 10^{10} s^{-1} for **1bH** as substrate and values of 1.9 and 1.8 × 10^{10} s^{-1} for **1cH** as substrate. Consistent with these values we have obtained k_{OH} = 2.2 × 10^{10} s^{-1} for bicyclo[2.1.0]pentane as substrate. Substrates such as methylcyclopropane and 1,1-dimethylcyclopropane, for which the corresponding cyclopropylmethyl radicals undergo relatively slow ring opening, yielded only the ring-closed alcohols on oxidation with cytochrome P-450. 1,1,2,2-Tetramethylcyclopropane gave only a trace of a ring-opened alcohol, corresponding to k_{OH} = 2.5 × 10^{11} s^{-1} for this substrate. Hexamethylcyclopropane gave no detectable ring-opened alcohol from which observation a limit for $k_{OH} > 5 \times 10^{11}$ s^{-1} can be calculated. Possible explanations for the unexpected behavior of these last two, relatively bulky, substrates are discussed.

The term cytochrome P-450 (or, more simply, P-450) refers to a family of membrane-bound, iron porphyrin (heme) containing monooxygenases which make use of molecular oxygen to catalyze the in vivo oxidation of a wide range of endogenous and exogenous compounds.[3] Specific isozymes may have extremely narrow or very broad substrate specificities.[3] One of the most intriguing of P-450s oxidations involves the hydroxylation of unactivated carbon–hydrogen bonds, that is the hydroxylation of alkanes, reaction 1.

Mechanistic studies of alkane hydroxylations with P-450 and with P-450 model catalysts have indicated that the reaction proceeds via the catalytic cycle shown in Scheme I.[4] Starting at the top left corner of this scheme with the enzyme in its Fe^{III} "resting state" the cycle proceeds through the following stages: (i) reversible substrate binding, (ii) one-electron reduction,[5] (iii) reversible binding of molecular oxygen, (iv) a second one-electron reduction[5] and the addition of two protons leads to the heterolytic cleavage of the O–O bond, a process which "unmasks" the activated catalytic species and, finally (v) reaction of the activated oxygen/substrate complex to yield the alcohol product and to regenerate the resting state P-450.

Our interest is in stage (v) of this catalytic cycle in which the high-spin oxoiron(IV) porphyrinate species,[3,6] [Fe^{IV}=O], and the

Scheme I



enzyme-bound substrate undergo reaction. This process has been extensively investigated with P-450 (and with P-450 mimic) catalysts.[3] Early mechanistic studies suggested that oxygen atom delivery to the substrate occurred via a concerted *oxene insertion* mechanism.[8] Thus, in many cases hydroxylation occurred with essentially complete stereoselectivity.[9-11] Furthermore, primary *inter*molecular deuterium kinetic isotope effects were generally found to have small values ($k_H/k_D \leq 2.0$)[9-15] and in some cases

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(2) NRCC Research Associate 1988-1990.
(3) *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum: New York, 1986.
(4) For a detailed discussion of oxygen activation by P-450 see ref 3: Ortiz de Montellano, P. R. pp 217-271; McMurry, T. J.; Groves, J. T. pp 1-28. See, also: Dawson, J. H. *Science* **1988**, *240*, 433-439. Mansuy, D.; Battioni, P. In *Activation and Functionalization of Alkanes*; Hill, C. L., Ed.; Wiley-Interscience: New York, 1989; Chapter 6.
(5) This electron is provided by the enzyme, cytochrome P-450 reductase which itself is reduced by NADPH.[3,4]
(6) This species is believed to be a π radical cationic oxoiron(IV) porphyrinate[3] which we symbolize by O=>Fe<•+. Spectroscopic data have confirmed this structure for an analogous "oxenoid" species prepared from a P-450 model catalyst.[7]
(7) Mandon, D.; Weiss, R.; Franke, M.; Bill, E.; Trautwein, A. X. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 1709-1711.
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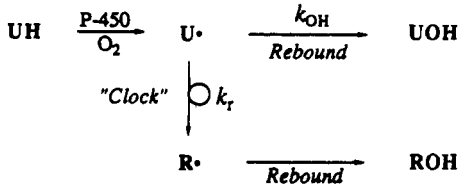
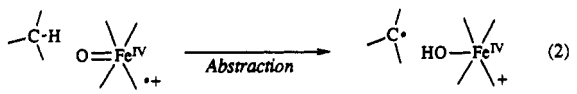
Scheme II



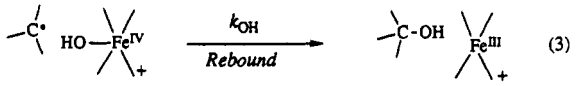
no isotope effect could be detected (k_H/k_D = 1.0).[11,12,16,17]

The generally negligible magnitude of *inter*molecular deuterium kinetic isotope effects was usually taken to imply that there was little C–H bond stretching in the transition state for hydroxylation. However, this conclusion rests on the assumption that step (v) is rate controlling in the overall catalytic cycle. That this is not the case has been clearly demonstrated by the observation of large primary *intra*molecular deuterium kinetic isotope effects (k_H/k_D = 7–14) with a variety of substrates.[15,18-26] Furthermore, a significant loss of stereochemistry was observed in the hydroxylation of (exo-d_4) labeled norbornane.[19] In addition, in the hydroxylation of appropriately labeled (with chlorine[27] or deuterium[28]) cyclohexene there is a partial loss of regioselectivity, i.e., among the products are cyclohexenols which have undergone an allylic rearrangement.

The currently accepted mechanism for step (v) of the P-450 catalytic cycle for alkane hydroxylation is based on the foregoing experimental observations.[3,29] Two discrete reactions are postulated: first, abstraction of a hydrogen atom from the substrate by the oxoiron species, reaction 2, and second, a rapid collapse of the resulting carbon-centered radical/hydroxyferryl complex to give the product alcohol and the resting state enzyme, reaction 3.

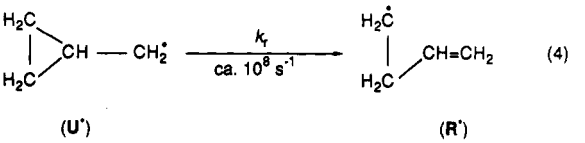


(2)



(3)

This, the *oxygen-rebound* mechanism, was originally proposed by Groves et al.[19] and has since been extended to hydroxylations mediated by nonenzymic iron porphyrin catalysts where it nicely accounts for the observation that some carbon-centered radicals "escape" hydroxylation and can be captured by solvents such as CCl_3Br.[30] Retention of configuration in many hydroxylations mediated by hepatic strains of P-450 implies an extremely rapid *rebound* step, reaction 3.[31] In an attempt to check the mechanism and, coincidentally, measure the rate constant, k_{OH}, for reaction 3, Ortiz de Montellano and Stearns[42] examined the rat hepatic microsomal hydroxylation of methylcyclopropane. The corresponding cyclopropylmethyl radical (**U•** in Scheme II) was known to rearrange by ring opening to the 3-butenyl radical (**R•**), reaction 4, with a rate constant k_r of ca. 10^8 s^{-1}.[43,44] Thus, methylcyclopropane provides a *calibrated* free-radical "*clock*"[44] with which the rate of *oxygen rebound* might be determined from the relative yields of the two alcohols (see Scheme II). In the event, the P-450 hydroxylation afforded only cyclopropanemethanol[42] implying that $k_{OH} \gg 10^8$ s^{-1}.[45]



(4)

(U•) (R•)

Bicyclo[2.1.0]pentane was also subjected to P-450-catalyzed hydroxylation by Ortiz de Montellano and Stearns[42] because this

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(29) Quadricyclane appears to be an exception in that it is oxidized by P-450 first to a radical cation which is captured in a second step by the activated oxygen species, see: Stearns, R. A.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1985**, *107*, 4081–4082.
(30) Groves, J. T.; Nemo, T. E. *J. Am. Chem. Soc.* **1983**, *105*, 6243–6248. Note that alkyl radicals may have been identified via spin trapping with nitrones during the P-450-catalyzed oxidation of 4-alkyl-3,5-bis(ethoxycarbonyl)-2,6-dimethyl-1,4-dihydropyridines. See: Augusto, O.; Beilan, H. S.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1982**, *257*, 11288–11295. Lee, J. S.; Jacobsen, N. E.; Ortiz de Montellano, P. R. *Biochemistry* **1988**, *27*, 7703–7710. For reviews of radical formation during P-450-catalyzed reactions, see: Dolphin, D. *Basic Life Sci.* **1988**, *49*, 491–500. Ortiz de Montellano, P. R. *Trends Pharmacol. Sci.* **1989**, *10*, 354–359. For a contrary view, see: Kennedy, C. H.; Mason, R. P. *J. Biol. Chem.* **1990**, *265*, 11425–11428.
(31) The alternative of a very tight and selective substrate binding seems unlikely because phenobarbital induced hepatic microsomes and purified phenobarbital induced hepatic strains of P-450 such as P-450_{LM2} (rabbit) and P-450_b (rat) exhibit reduced substrate selectivities relative to uninduced microsomes,[9,22,32-34] although exceptions to this general rule have been reported.[37] Furthermore, substrate size vs binding energies[38,39] and other evidence[40] indicate that small aliphatic substrates are bound to these types of P-450 in relatively capacious sites by weak hydrophobic forces.
(32) McMahon, R. E.; Sullivan, H. R. *Life Sci.* **1966**, *5*, 921–926.
(33) Lu, A. Y. H.; West, S. B. *Pharmacol. Rev.* **1980**, *31*, 277–291.
(34) For example,[35] *n*-hexane is oxidized to 1-, 2-, and 3-hexanol in a ratio of 1:3:0.5, respectively, by uninduced rat liver microsomes and to the same three products in a ratio of 1:13:3, respectively, by phenobarbital-induced rat liver microsomes. Similarly,[36] *n*-heptane is oxidized to 1-, 2-, 3-, and 4-heptanol in a ratio of 1:5:0.8:0.2, respectively, by uninduced rat liver microsomes and to the same four products in a ratio of 1:13:2.5:0.5, respectively, by phenobarbital-induced rat liver microsomes. Again,[22] *n*-octane is hydroxylated to 1-, 2-, and 3-octanol in a ratio of 1:5:0.6 by phenobarbital-induced rat liver microsomes, while P-450_b yields the same products in a ratio of 1:23:7. Note that the hydroxylation of the primary carbon of *n*-octane by uninduced rat liver microsomes[41a] and the primary allylic carbon of geraniol by microsomal P-450 from *Catharanthus roseus* (L.) G. DON[41b] both proceed with retention of configuration.
(35) Frommer, U.; Ullrich, V.; Orrenius, S. *FEBS Lett.* **1974**, *41*, 14–16.
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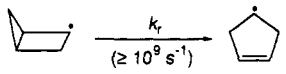
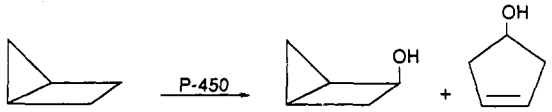
(44) Griller, D.; Ingold, K. U. *Acc. Chem. Res.* **1980**, *13*, 317–323.
(45) Another possibility could be that the reaction is concerted so that a carbon-centered radical is not involved.[42]

hydrocarbon would yield a radical which was known from studies by Jamieson et al.[46] to rearrange more rapidly than cyclopropylmethyl, though the rate of this ring opening had proved too fast ($k_r \geq 10^9\ s^{-1}$) to measure by the EPR method.[46] The P-450 hydroxylation gave a 7:1 ratio of unrearranged to rearranged



(5)

alcohols,[42] reaction 6, implying that oxygen rebound is about seven times faster than the ring-opening of the bicyclo[2.1.0]pent-2-yl radical. In a preliminary communication,[47] we reported on the *calibration* of this radical *clock* by the nitroxide radical trapping



(7) (1) (6)

(NRT) technique[47,48] (vide infra) and gave a *first* estimate of k_{OH} of ca. $2 \times 10^{10}\ s^{-1}$ for the bicyclo[2.1.0]pentane/P-450 combination. In the present paper we refine this estimate and, more importantly, we make use of the extensive kinetic data we have obtained by NRT *calibration* of the ring-opening rates for the radicals derived from symmetrically substituted polymethylcyclopropanes[48] to investigate *oxygen rebound* and determine k_{OH} for these molecules as P-450 substrates. Our results with some substrates are very surprising and should serve as a warning which, hopefully, will prevent the uncritical use of radical clocks as probes of enzyme reaction mechanisms and kinetics.

Experimental Section

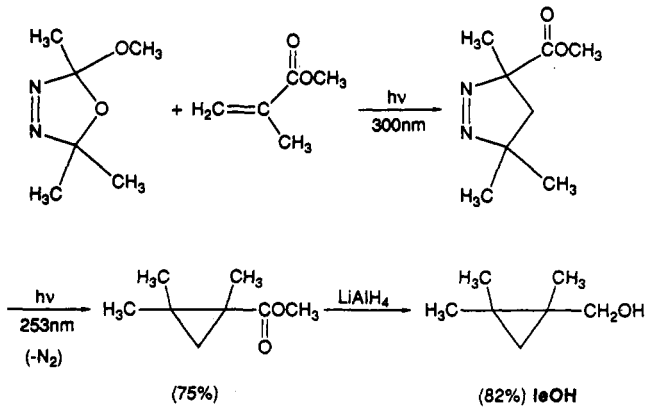
Instrumentation. Product analyses were by GC/MS using a Hewlett-Packard HP 5890 GC instrument and a cross-linked methyl silicon column (HP 1, 12 m × 0.2 mm i.d.) interfaced with either an HP 5970 (EI, 70 eV) mass selective detector or an HP 5988A mass spectrometer (CI (CH_4), 120 eV), each of them linked with an HP 59970C MS data analyses system. NMR spectra were recorded on Bruker AM 400 (400 MHz for ^1H and 61 MHz for ^2H) or Varian EM 360 (60 MHz) instruments with $CDCl_3$ as solvent and tetramethylsilane as an internal standard. UV absorptions were recorded on an HP 8450 B diode array spectrophotometer.

Materials. For ready reference the compounds used in this work have been given the same code numbering system as in the preceding paper.[48]

(1) Hydrocarbons. The following compounds were commercially available and were used, as received, as substrates for P-450-catalyzed hydroxylation: methylcyclopropane, **1aH** (API, PA); *trans*-1,2-dimethylcyclopropane, **1bH** (Wiley, OH); *cis*-1,2-dimethylcyclopropane, **1cH** (Wiley); 1,1-dimethylcyclopropane, **1dH** (Wiley); 1,1,2,2-tetramethylcyclopropane, **1eH** (Wiley); and benzylcyclopropane, **1lH** (Lancaster Synthesis, NH). In addition, hexamethylcyclopropane,[48,49] **1fH**; bicyclo[2.1.0]pentane,[42,48,50] **4H**; *cis*-2,3-dideuteriobicyclo[2.1.0]pentane,[42,48] ***d*$_2$4H** (*exo-cis*/*endo-cis*-[$^2H_2$]-bicyclo[2.1.0]pentane ≈ 2.0/1 by ^2H NMR); and dispiro[2.2.2.2]decane,[48,51] **1kH**; were synthesized by published procedures and used as P-450 substrates. 2,3,3,4-Tetramethyl-1,4-pentadiene (a potential product from the P-450 catalyzed oxidation of **1fH** (vide infra), was also synthesized by a published procedure.[52]

(2) Alcohols. The following alcohols were purchased from Aldrich, WI, for use as analytical standards: cyclopropanemethanol, **1aOH**; 3-buten-1-ol, **2aOH**; *trans*-2-methylcyclopropanemethanol, **1bOH** (as a ca. 85%:15% mixture with the cis isomer, **1cOH**); 4-penten-2-ol, **2bOH**; 2-methyl-3-buten-1-ol, **3bOH**; 1-methylcyclopropanemethanol, **1dOH**; 3-methyl-3-buten-1-ol, **2dOH**; and α-cyclopropylbenzyl alcohol, **1lOH**. In addition, 1,1,3-trimethylbut-3-en-1-ol, **2eOH**, was purchased from Wiley, and the following alcohols were synthesized by the procedures indicated.

Scheme III



(i) cis-2-Methylcyclopropanemethanol,[53] 1cOH, was prepared in order to have an unequivocal identification of the trans and cis isomers present in the commercial material, vide supra. Lindlar hydrogenation[54] (34 psig H_2 for 1 h) of 2-butyn-1-ol gave 2-buten-1-ol (86%) which, on acetylation (acetyl chloride/pyridine) and cyclopropanation (1.5 mol equiv of Simmons–Smith reagent[55] for 5 h at reflux; yield 66%), and saponification gave **1cOH** in an overall yield of 35%, bp 62–64 °C at 100 mmHg: ^1H NMR δ (ppm) 0.5–1.1 (m, 4 H), 1.05 (d, 3 H, J = 3 Hz), 2.2 (s, 1 H), 3.6 (m, 2 H).

(ii) 2,2,3-Trimethyl-3-buten-1-ol, 3eOH, was prepared via a standard methylenation[56] of ethyl dimethylacetoacetate (Shaunee Chemicals, OH) with 1.5 mol equiv of $(C_6H_5)_3PCH_3^+Br^-$/NaH for 2 h at 75 °C to obtain ethyl 2,2,3-trimethyl-3-butenoate (60% conversion, 40% isolated yield) followed by treatment[57] with $LiAlH_4$ to afford **3eOH** which was purified by flash chromatography on alumina: ^1H NMR δ (ppm) 1.1 (s, 6 H), 1.6 (s, 3 H), 3.4 (s, 2 H), 4.7 (m, 1 H), 4.8 (m, 1 H).

(iii) 2,3,3,4-Tetramethyl-4-penten-2-ol, 2fOH, was prepared by reaction of ethyl 2,2,3-trimethyl-3-butenoate (vide supra) with MeMgI (3 mol equiv) for 5 h in ether at reflux and was purified by flash chromatography on alumina: ^1H NMR δ (ppm) 1.1 (s, 6 H), 1.15 (s, 6 H), 1.85 (s, 3 H), 4.7 (m, 1 H), 4.9 (m, 1 H); GC/MS (EI) retention time, 5.8 min; *m/e* (rel intensity), 142 (M^+, 0), 127 (2), 124 (1), 109 (17), 84 (51), 69 (87), 67 (20), 59 (100), 55 (20).

(iv) 1,1,2-Trimethylcyclopropanemethanol,[58] 1eOH, was prepared by $LiAlH_4$ reduction[57] of methyl 1,2,2-trimethylcyclopropanecarboxylate which itself had been prepared by photolysis (253 nm, 5 h) of a 2% pentane solution of a generous gift of 3-methoxy-3,5,5-trimethyl-4-oxa-1-pyrazoline (see Scheme III and Acknowledgments): ^1H NMR δ (ppm) 0.2 (d, 1 H, J = 5 Hz), 0.3 (d, 1 H, J = 5 Hz), 1.1 (s, 3 H), 1.15 (s, 3 H), 1.2 (s, 3 H), 3.5 (d, 1 H, J = 11 Hz), 3.65 (d, 1 H, J = 11 Hz), 3.9 (s, 1 H).

(v) 4-Phenyl-3-buten-1-ol, 2lOH, was prepared by $LiAlH_4$ reduction[57] of 4-phenyl-3-butenoic acid (Aldrich): ^1H NMR δ (ppm) 2.3–2.4 (m, 3 H), 3.3 (t, 2 H, J = 7 Hz), 6.2 (m, 1 H), 6.3 (d, 1 H, J = 15 Hz), 7.1–7.5 (m, 5 H).

(vi) 3-Cyclopenten-1-ol, 5OH, was prepared from cyclopentadiene by a published procedure:[59] ^1H NMR δ (ppm) 2.0–2.5 (m, 4 H), 2.8 (s, 1 H), 4.45 (septet, 1 H), 5.7 (s, 2 H).

Finally, the following compounds were identified or were shown to be absent, as indicated. 1,2,2,3,3-Pentamethylcyclopropanemethanol, **1fOH**, was shown to be the only alcohol product in the extract of the **1fH** incubate by GC/MS by using both CI and EI with ion-selective monitoring: GC/MS (EI) retention time, 6.7 min; *m/e* (rel intensity), 142 (M^+, 0), 125 (6), 124 (6), 109 (100), 81 (23), 67 (83), 55 (60). *endo*-Bicyclo[2.1.0]pentan-2-ol, **4OH**,[60] was identified by comparison with the

(46) Jamieson, C.; Walton, J. C.; Ingold, K. U. *J. Chem. Soc., Perkin Trans. 2* **1980**, 1366–1371.
(47) Bowry, V. W.; Lusztyk, J.; Ingold, K. U. *J. Am. Chem. Soc.* **1989**, *111*, 1927–1928.
(48) Bowry, V. W.; Lusztyk, J.; Ingold, K. U. *J. Am. Chem. Soc.*, preceding paper in this issue.
(49) Fischer, P.; Schaefer, G. *Angew. Chem. Int. Ed. Engl.* **1981**, *20*, 863–864.
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(55) LeGoff, E. *J. Org. Chem.* **1964**, *29*, 2048–2050.
(56) Greenwald, R.; Chaykovsky, M.; Corey, E. J. *J. Org. Chem.* **1963**, *28*, 1128–1129.
(57) McMurry, J. E. *Organic Syntheses*; Wiley: New York, 1973; Vol. 53, pp 70–71.
(58) Allred, E. L.; Flynn, C. R. *J. Am. Chem. Soc.* **1975**, *97*, 614–621.
(59) Cremer, S. E.; Blankenship, C. *J. Org. Chem.* **1982**, *47*, 1629–1632.

GC retention time and EI mass spectrum reported for this compound by Ortiz de Montellano and Stearns.[42] A search by CG/MS using both CI and EI with ion-selective monitoring showed that neither of the two potential alcohol products from dispiro[2.2.2.2]decane, **1kH**, viz., **1kOH** and **2kOH** (vide infra, eq 13) were present in detectable quantities in the **1kH** microsomal incubate.

(3) Microsomes. The preparation of hepatic microsomes from phenobarbital-pretreated male Sprague–Dawley rats followed the procedure described by Ortiz de Montellano et al.[61] in detail. Briefly, the rats were injected intraperitoneally once a day for 4 consecutive days with phenobarbital as its sodium salt in water (80 mg/Kg body weight) and were sacrificed on the fifth day. Their livers were perfused in situ with ice-cold isotonic KCl solution (0.15 M), excised, and used to prepare a 33–50% (w/v) homogenate in isotonic KCl. This homogenate was centrifuged, and, following the described procedure,[61] a washed-microsome pellet was obtained which was then suspended in 0.10 M Na/K-phosphate buffer (pH 7.4) containing KCl (0.15 M) and EDTA (0.15 mM). These microsome suspensions were then diluted with isotonic KCl to a microsomal P-450 content of 16 nM/mL. The microsomal P-450 content was determined as described by Estabrook et al.[62] from the difference in the visible absorption spectra of suitably diluted (ca. 5-fold), carbon monoxide-saturated suspensions which had and had not been reduced by the addition of a few crystals of sodium dithionite ($Na_2S_2O_4$). The P-450 concentration was estimated from the standard corrected molar absorptivity difference: $\Delta\Sigma_{450nm} - \Delta\Sigma_{490nm} = 1 \times 10^5$ [P-450]$^{-1}$ M^{-1} cm^{-1}.[62] This microsomal suspension was stored in small vials at −80 °C, such storage having no observable effect on the catalytic or spectral properties of the microsomes.

Phenobarbital induces several P-450 isozymes in rat hepatic microsomes.[3,39,63–65] The two major isozymes obtained from male Sprague–Dawley rats (the rats used in the present study) are P-450$_b$ (which is also referred to as P-450$_{PB-4}$ or, more formally,[66] as P-450 IIB1) and P-450$_e$ (which is also referred to as P-450$_{PB-5}$ or, more formally,[66] as P-450 IIB2).

Reaction Procedures. For liquid substrates the freshly thawed microsome suspension (4 mL, 16 nM) was diluted with potassium phosphate buffer (12 mL, 0.10 M in phosphate, pH 7.4) containing EDTA (1.5 mM) and KCl (0.15 M) and placed in a scintillation vial capped and sealed with a rubber septum. The liquid substrate (10 μL) was injected through the septum, and the mixture was shaken for 5 min at 37 °C in a thermostated water bath. Activation of the microsomal P-450 was then achieved by injection of NADPH (Sigma, MO) (1.2×10^{-5} mol in 0.5 mL buffer) and incubated at 37 °C for appropriate periods of time (see tables). Reactions were stopped by rapid cooling in ice, and, after the addition of a suitable GC standard[67] the incubate was extracted with diethyl ether[68] (2 × 15 mL), the extract was dried over Na_2SO_4 and then concentrated by careful evaporation of the solvent through a short Vigreux column to a final volume of ca. 0.3 mL.

For the gaseous substrate, methylcyclopropane, the NADPH activated, buffered microsome suspension was placed in an ampoule connected to a high vacuum system and frozen with liquid nitrogen. By standard vacuum transfer techniques 0.1 mmol of methylcyclopropane was then added, followed by air to a pressure of ca. 200 Torr. The ampoule was flame sealed and then shaken in the water bath at 37 °C for 30 min. Product workup followed the same procedure as outlined above except that extra care was taken in concentrating the ether extract in order to avoid losing the alcohol product(s).

The concentrated organic extracts were analyzed by coupled gas chromatography/mass spectrometry (GC/MS) with greater sensitivity and more uniform detection of products being achieved with chemical ionization (CI) than with electron impact ionization (EI).[69] A moderate GC injection port temperature of 100 °C and low column temperatures were employed to avoid rearrangement and/or dehydration of heat-sensitive alcohols, viz., 30 °C increasing after 4 min to 240 °C at 15 °C/min for most incubate extracts but −20 °C increasing after 5 min to 240 °C at 15 °C/min for the methylcyclopropane incubate extract. In all cases, raising the injection port temperature to 130 °C had no observable effect on alcohol product ratios.

The products were identified by comparison with the GC/MS data for authentic materials except for **1fOH** and *endo*-**4OH** (vide supra). Relative alcohol yields in the incubate extracts were calibrated by using the response factors measured for similarly constituted mixtures of authentic materials. Extracts of blank reactions in which either the NADPH or the substrate had been omitted contained the usual mixture of lipids and other ether soluble microsomal materials but did not contain alcohols in detectable amounts.

Table I. Relative Product Yields and "Rebound Rate Constants (k_{OH})" for Radical Clock Substrates (**UH → UOH + ROH**)

U• → R•	$k^{37°}$(10^8 s^{-1})	Products[a] [UOH]:[ROH]	"k_{OH}" (s^{-1})
1a• → **2a•**	1.2	> 100:1	> 1 x 10^{10}
1b• → **2b•**	1.6	95 (±16):1	1.5 x 10^{10}
1b• → **3b•**	1.8	90 (±15):1	1.6 x 10^{10}
1c• → **2b•**	8.0	24 (±5):1	1.9 x 10^{10}
1c• → **3b•**	2.3	77 (±9):1	1.8 x 10^{10}
1d• → **2d•**	0.8	> 100:1	> 1 x 10^{10}
1e• → **2e•**	20	100:0.8	2.5 x 10^{11}
1e• → **3e•**	2.1	> 200:1	> 5 x 10^{10}
1f• → **2f•**	47	> 100:1	> 5 x 10^{11}
1k• → **2k•**	4.1	No reaction with P-450	
1l• ⇌ **2l•**	≤0.01 / 0.1	>200:1	>10^8
4•, $d_2$4• → **5•, $d_2$5•**	21	10.5:1[c,d] (7:1)[e]	2.2 x 10^{10}

[a] Incubation times were 30 min (after NADPH addition) except for **1eOH** (20 min) and **4H** (see text and Table II). [b] The dideuterated material was a 2.0:1 mixture of *exo*- and *endo-cis*-2,3-[$^2H_2$]-bicyclo-[2.1.0]pentane, see text. [c] Product ratios were not affected by deuteration. [d] Extrapolated to zero reaction time (see text). [e] Reference 42.

Results

Methylcyclopropane, 1aH. In agreement with the result reported by Ortiz de Montellano and Stearns[42] the only alcohol product that could be detected in the P-450-catalyzed oxidation of **1aH** was cyclopropanemethanol, **1aOH**. A selected ion search

(60) Wieberg, K. B.; Barth, D. E. *J. Am. Chem. Soc.* **1969**, *91*, 5124–5130.
(61) Ortiz de Montellano, P. R.; Mico, B. A.; Mathews, J. M.; Kunze, K. L.; Miwa, G. T.; Lu, A. Y. H. *Arch. Biochem. Biophys.* **1981**, *210*, 717–728.
(62) Estabrook, R. W.; Peterson, J.; Baron, J.; Hildebrandt, A. In *Methods in Pharmacology*; Chignell, C. F., Ed.; Appleton-Century-Crofts: New York, 1972; Vol. 2, pp 303–350.
(63) Waxman, D. J. ref 3, pp 525–539.
(64) Ryan, D. E.; Thomas, P. E.; Reik, L. M.; Levin, W. *Xenobiotica* **1982**, *12*, 727–744. See, also: Fujii-Kuriyama, Y.; Mizukami, Y.; Kawajiri, K.; Sogawa, K.; Muramatsu, M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 2793–2797.
(65) Waxman, D. J.; Walsh, C. *J. Biol. Chem.* **1982**, *257*, 10446–10457.
(66) Gonzales, F. J. *Pharmac. Ther.* **1990**, *45*, 1–38.
(67) Standards were chosen to match, more or less, the volatility and water solubility of the product alcohols, e.g., 2-butanol for the methylcyclopropane reaction, 1-heptanol for the 1,1,2,2-tetramethylcyclopropane reaction, etc.
(68) More volatile ethers which would have made concentrating the product an easier task were too miscible with water.
(69) This is because EI gives more complex fragmentation patterns than CI.

for 3-buten-1-ol, **2aOH**, in the GC/MS of the extracted products indicated that, if present, its concentration must be at least 100-fold less than that of the unrearranged alcohol, **1aOH**. This information is summarized in Table I together with the rate constant, k_r, for the **1a**$^{\bullet}$ → **2a**$^{\bullet}$ rearrangement at 37 °C (see preceding paper)[48] and the calculated *lower limit* for the rate constant for *oxygen rebound*, k_{OH}, with methylcyclopropane as the *clock* substrate.

***trans*-1,2-Dimethylcyclopropane, 1bH.** The corresponding radical, **1b**$^{\bullet}$, undergoes ring opening to yield both the thermodynamically favored secondary pent-1-en-4-yl radical, **2b**$^{\bullet}$, and the thermodynamically disfavored primary 3-methylbut-1-en-4-yl radical, **3b**$^{\bullet}$.[46] These two product radicals are formed in approximately equal amounts at 37 °C in homogeneous solution, and the rates of the two separate ring-opening reactions are ca. 50% greater than the overall rate of the **1a**$^{\bullet}$ → **2a**$^{\bullet}$ reaction.[48] The microsomal P-450 oxidation products from **1bH** consisted of *three* alcohols. By far the major product was the unrearranged alcohol, **1bOH**. The two "expected" rearranged alcohols, **2bOH** and **3bOH**, were minor products *which were formed in approximately equal amounts*. The relative yields of **1bOH**, **2bOH**, and **3bOH** did not vary with the duration of the incubation period (15, 30, and 45 min) but may have varied when different batches of microsomes were employed. Thus, for three different microsome preparations (batches 1, 2, and 3) values of the **1bOH/2bOH** ratio from these preparations were 92 (batch 1, 15 min), 90 ± 6 (batch 1, 30 min, three experiments), 101 (batch 1, 45 min), 76 ± 11 (batch 2, 30 min, three experiments), and 116 ± 10 (batch 3, 30 min, three experiments), while the corresponding **1bOH/3bOH** ratios were 85 (batch 1, 15 min), 87 ± 8 (batch 1, 30 min, three experiments), 88 (batch 1, 45 min), 82 ± 8 (batch 2, 30 min, three experiments), and 102 ± 9 (batch 3, 30 min, three experiments). For **1bH** as substrate each of the two product ratios given in Table I are averages (±1 standard deviation) of all individual experimental measurements. As would be expected from our NRT measurements[48] the averaged **1bOH/2bOH** ratio is slightly larger than the averaged **1bOH/3bOH** ratio. Both product ratios can be combined with the appropriate ring-opening rate constant to calculate k_{OH}. The two values that are obtained for k_{OH}, viz., 1.5 and 1.6 × 10^{10} s^{-1} (see Table I), are in excellent agreement with each other.

***cis*-1,2-Dimethylcyclopropane, 1cH.** This substrate also gave the three alcohols "expected", viz., **1cOH**, **2bOH**, and **3bOH**. Again the product ratios did not depend on the duration of the incubation period (15, 30, and 45 min), but they may have varied from one batch of microsomes to another. For the same three microsome preparations values of the **1cOH/2bOH** ratio were 20 (batch 1, 15 min), 23 ± 5 (batch 1, 30 min, three experiments), 18 (batch 1, 45 min), 20 ± 2 (batch 2, 30 min, three experiments), and 30 ± 8 (batch 3, 30 min, three experiments), while the corresponding **1cOH/3bOH** ratios were 81 (batch 1, 15 min), 87 ± 16 (batch 1, 30 min, three experiments), 72 (batch 1, 45 min), 75 ± 12 (batch 2, 30 min, three experiments), and 69 ± 11 (batch 3, 30 min, three experiments). Average ratios (±σ) are given in Table I together with the k_{OH} values calculated by combining these ratios with the NRT measured[48] rates of the two ring-opening reactions. Again the two values that are obtained for k_{OH}, viz., 1.9 and 1.8 × 10^{10} s^{-1}, are in excellent agreement with each other and are in gratifying agreement with the values calculated from the **1bH** data (see Table I).

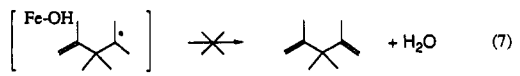
1,1-Dimethylcyclopropane, 1dH. The radical **1d**$^{\bullet}$ undergoes ring opening at only ca. $^2/_3$ of the rate of ring opening of the cyclopropylmethyl radical, **1a**$^{\bullet}$.[48] As would be expected therefore on the basis of results with **1aH** (ref 42 and Table I), the incubation of **1dH** with microsomes yielded only the unrearranged alcohol, **1dOH**. If the ring-opened alcohol, **2dOH**, was present, a selected ion search in the CG/MS indicated that its concentration was <1% the concentration of **1dOH**. This allows us only to say that for **1dH** as substrate, k_{OH} > 10^{10} s^{-1} (see Table I).

1,1,2,2-Tetramethylcyclopropane, 1eH. Our initial incubation of this hydrocarbon with the microsomes (and one subsequent incubation) afforded a mixture of the unrearranged alcohol, **1eOH**, and the tertiary ring-opened alcohol, **2eOH**, in an ca. 3:1 ratio. However, *none* of the primary ring-opened alcohol, **3eOH**, could be detected, and selected ion monitoring indicated that if this alcohol was present in the incubate its concentration was <0.5% that of the unrearranged alcohol. This last result is inconsistent with a relative yield of **3eOH** of ca. 3% that could be calculated from the "observed" **1eOH/2eOH** ratio of 3:1 and the NRT measured rate constants[48] for the two modes of ring opening. For this reason, **1eH** was incubated for various lengths of time (5–240 min) with an additional ten batches of microsomes. In all of these experiments (11 in all) we succeeded in detecting just a trace (ca. 0.6–0.8%) of the tertiary alcohol, **2eOH**, but could find no trace of the primary alcohol, **3eOH**. We attribute the (nonrepeatable) detection of **2eOH** in two experiments to its formation during workup via a polar rearrangement of **1eOH** induced by some adventitious impurity.

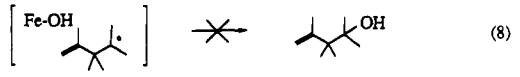
The unrearranged alcohol, **1eOH**, is oxidized to the corresponding aldehyde during the incubation. The yields of aldehyde and rearranged tertiary alcohol, **2eOH**, as a percentage of the yield of **1eOH** were, for example, 2.9 and 0.8% after 5-min incubation, 3.9 and 0.8% after 10-min incubation, 6.8 and 0.8% (7.3 and 0.6%) after 120-min incubation, and 11.2 and 0.8% after 240-min incubation. Taking the **1eOH/2eOH** ratio to be 100:0.8 yields k_{OH} = 2.5 × 10^{11} s^{-1} for **1eH** as the clock substrate (see Table I). Our failure to detect the primary rearranged alcohol, **3eOH**, allows us also to put an apparent lower limit on k_{OH} of 5 × 10^{10} s^{-1} (see Table I).

Hexamethylcyclopropane, 1fH. Despite the rapidity with which **1f**$^{\bullet}$ undergoes ring opening[48] we could detect only a single alcohol in the two batches of microsomes examined. This alcohol was identified as the unrearranged product, **1fOH**. Assuming that we could have detected ca. 1% of the rearranged alcohol, **2fOH**, this result puts an apparent lower limit[70] on k_{OH} of 5 × 10^{11} s^{-1} (Table I).

The possibility that **2f**$^{\bullet}$ reacts with the hydroxyferryl complex by disproportionation (reaction 7) rather than by oxygen rebound (reaction 8) was also explored since such a mechanism has been

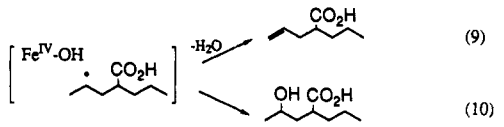


(7)



(8)

suggested to explain, for example,[71] the desaturation of valproic acid by microsomes (particularly by phenobarbital induced strains), cf. reactions 9 and 10. However, a careful search by GC/MS with selective ion monitoring for 2,3,3,4-tetramethyl-



(9)
(10)

1,4-pentadiene (the only possible ring-opening/disproportionation product, see reaction 7) failed to reveal any trace of this compound (<0.5% based on the yield of **1fOH**). We are therefore forced to conclude that **1fH** does not give rise to any ring-opened oxidation products.

Dispiro[2.2.2.2]decane, 1kH. The radical formed by hydrogen atom abstraction from this compound undergoes very rapid ring opening because of favorable stereoelectronic factors,[48] which suggested that **1kH** would be a useful clock substrate for measuring k_{OH}. Unfortunately, this hydrocarbon was not oxidized by

(70) An attempt to refine the sensitivity for detection of the tertiary alcohol by trimethylsilylation was not successful.

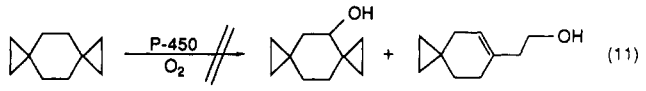
(71) Rettie, A. E.; Boberg, M.; Rettenmeier, A. W.; Baillie, T. A. *J. Biol. Chem.* **1988**, *263*, 13733–13738. See, also: Wood, A. W.; Swinney, D. C.; Thomas, P. E.; Ryan, D. E.; Hall, P. F.; Levin, W.; Garland, W. A. *J. Biol. Chem.* **1988**, *263*, 17322–17332. Nagata, K.; Liberato, K. D.; Gillette, J. R.; Sesame, H. A. *Drug Metab. Dispos.* **1986**, *14*, 559–565.

Table II. Time Dependence of the *endo*-Bicyclo[2.1.0]pentan-2-ol (**4OH**) to 3-Cyclopentenol (**5OH**) Ratio at 37 °C

time (min)	0	15	30	60	120
[**4OH**]/[**5OH**][a]		9.2	8.0	7.0	
[**4OH**]/[**5OH**][b]	8.0	6.1	5.1	4.4	2.9

[a] From the microsome reaction mixture. [b] The "30 min" reaction product in phosphate buffer, pH 7.4.

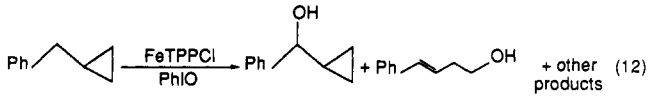
P-450, neither the unrearranged secondary alcohol, **1kOH**, nor the rearranged primary alcohol **2kOH** being present in detectable quantities in the incubates.



1kH **1kOH** **2kOH**

The absence of alcohol products is *not* due to an intrinsically low reactivity for the hydrogen atoms on the cyclohexane ring. In fact, toward *tert*-butoxyl radicals these eight hydrogens are, in total, 40 times as reactive as the three methyl hydrogens of methylcyclopropane.[48]

Benzylcyclopropane, 1lH. A recent report[72] that a trace (<0.5%) of the ring-opened alcohol, 4-phenylbut-3-en-1-ol, **2lOH**, is formed when **1lH** is oxidized by tetraphenylporphyrinatoiron(III) chloride (FeTPPCl, a P-450 model compound) and iodosobenzene, reaction 12, prompted us to investigate the reaction

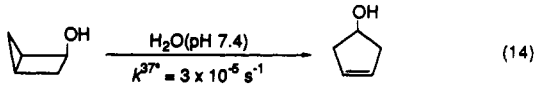


1lH **1lOH** (trace) **2lOH**

of this substrate with microsomal P-450. In the event, relatively rapid substrate turnover afforded the unrearranged alcohol, **1lOH**, reaction 13, as the *only* detectable alcohol product. Selective

ion searching of a chromatographycally purified sample of the P-450 metabolite indicated that the **1lOH**/**2lOH** ratio was >200 (see Table I).

Bicyclo[2.1.0]pentane, 4H. The P-450-catalyzed oxidation of this substrate afforded a mixture of *endo*-bicyclo[2.1.0]pentan-2-ol, **4OH**, and 3-cyclopenten-1-ol, **5OH**, as confirmed by comparison of our GC/MS (EI) data with published[42] GC and MS(EI) data for these two compounds. With a 30-min incubation time, the ratio of unrearranged to rearranged alcohols, i.e., **4OH**/**5OH**, was very similar to the value(s) reported (viz., 7.4:1, range 6:1 to 10:1) by Ortiz de Montellano and Stearns[42] for the same incubation time (see Table II). However, we found that the proportion of rearranged alcohol increased with an increase in the duration of the incubation period (see Table II). Moreover, when the "30-min extract" was treated with the buffer solution (pH 7.4) at 37 °C, the **4OH**/**5OH** ratio continued to decrease, and it decreased about twice as rapidly as in the P-450 reaction mixture (see Table II). These results are consistent with a polar rearrangement of the *endo*-bicyclo[2.1.0]pentan-2-ol subsequent to its formation by P-450 (reaction 14).[73-75]



4OH **5OH**

Extrapolation of the data in Table II to zero reaction time indicates that the true ratio of the P-450-catalyzed rates of formation of **4OH** vs **5OH** from **4H** is ca. 10.5. This ratio is just outside the range of values given by Ortiz de Montellano and Stearns[42] (vide supra), but it agrees extremely satisfactorily with their observation that **4OH** "is formed approximately 10 times faster" than **5OH**.

Our initial NRT measurements of the rate constant for the **4**• → **5**• rearrangement (reaction 5) gave $k_r = 2.4 \times 10^9\ s^{-1}$ at 37 °C.[47] However, this value had not been corrected for the fact that the rate constants for alkyl radical trapping decrease at high nitroxide concentrations.[48] After allowing for this effect, we estimate that $k_r = 2.1 \times 10^9\ s^{-1}$ at 37 °C,[48] a value which, when combined with the **4OH**/**5OH** product ratio of 10.5, yields $k_{OH} = 2.2 \times 10^{10}\ s^{-1}$ (see Table I).

***cis*-2,3-Dideuterobicyclo[2.1.0]pentane, $d_2$4H.** Ortiz de Montellano and Stearns[42] investigated the P-450-catalyzed oxidation of this compound in a mixture containing ca. 70% exo-d_2, ca. 20% endo-d_2, and ≤10% undeuterated **4H**. The deuterium distribution in the alcohol products was only consistent with removal of the endo hydrogen (or deuterium), and hydroxylation proceeded with complete retention of the stereochemistry, i.e., only *endo*-bicyclo[2.1.0]pentan-2-ol was formed. The substitution of deuterium for hydrogen, i.e., d_2**4H** for **4H**, did not alter the rate of product formation nor the ratio of unrearranged-to-rearranged alcohols.

Our preparation of d_2**4H** yielded an exo-d_2/endo-d_2 ratio of ca. 2.0/1.[76] In agreement with Ortiz de Montellano and Stearns,[42] the only unrearranged alcohol had the endo structure and the ratio of unrearranged-to-rearranged alcohols was quite unaffected by deuteration (i.e., extrapolation to zero reaction time gave a value of 10.5 for this ratio). However, the mass spectrum of the products indicated a d_2:d_1 ratio of 3.0(±0.4):1 in both the unrearranged and rearranged alcohols. This result, taken in conjunction with the known[42] stereospecific removal of an endo hydrogen or deuterium, implies the existence of a small *intermolecular* primary deuterium kinetic isotope effect in the range 1.3–1.7. The absence of exo alcohol despite the anticipated[15,18-26] rather large *intramolecular* isotope effect, indicates a remarkably high stereoselectivity for endo hydroxylation.

Suicide Inactivation of P-450. Ortiz de Montellano and Stearns[42] reported that the P-450 content of microsomes incubated with NADPH and methylcyclopropane, **1aH**, or bicyclo[2.1.0]pentane, **4H**, decreased in a time-dependent manner (by approximately 30% and 37%, respectively, in 30 min).[42] Since both NADPH and a substrate were required for P-450 to be destroyed, these are *mechanism-based* (or *suicide*) deactivations.[77-79] In preliminary experiments, we have confirmed their observation with regard to **4H**. Our results on the inactivation of P-450 by alkanes will be reported when additional work has been completed.

Discussion

Oxygen Rebound for "Well-Behaved" Clock Substrates. The data summarized in Table I will be considered first in terms of the six well-behaved clock substrates, viz., methylcyclopropane, **1aH**, the three dimethylcyclopropanes, **1bH**, **1cH**, and **1dH**, benzylcyclopropane, **1lH**, and bicyclo[2.1.0]pentane, **4H** (and its cis dideuterated isotopomer, d_2**4H**).

The production of rearranged alcohol products from **1bH**, **1cH**, and **4H** proves that these P-450-catalyzed processes are not concerted. Furthermore, the oxidation of **1bH** and **1cH** must occur via free-radical intermediates since cationic intermediates would

(72) Inchley, P.; Smith, J. R. L.; Lower, R. J. *New J. Chem.* **1989**, *13*, 669–676.

(73) This reaction has not been previously reported. However, we note that *endo*-bicyclo[2.1.0]pent-2-yl-3,5-dinitrobenzoate undergoes extremely rapid solvolysis[74] which indicates a very high S_N1 activity for the 2-endo position of 2-endo-substituted bicyclo[2.1.0]pentanes.

(74) Wiberg, K. B.; Williams, V. Z., Jr.; Friedrich, L. E. *J. Am. Chem. Soc.* **1968**, *90*, 5338–5339.

(75) Ortiz de Montellano and Stearns[42] reported a ca. 3% conversion of **4OH** to **5OH** under their GC conditions and hence concluded that this thermal rearrangement could be neglected in their quantitative studies of the **4H** oxidation products. This thermal rearrangement is also estimated to be of negligible importance under the analytical conditions used in the present work since the measured **4OH**/**5OH** ratios were not altered when the standard GC conditions (see Experimental Section) were changed.

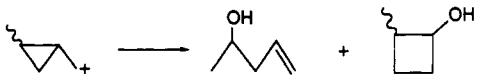
(76) Cf.: 70% exo-d_2, 20% endo-d_2, and ≤10% undeuterated alcohol.[42]

(77) Ortiz de Montellano, P. R.; Reich, N. O. ref 3, pp 273–314.

(78) Walsh, C. *Tetrahedron* **1982**, *38*, 871–909.

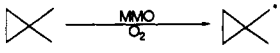
(79) Santi, D. V.; Kenyon, G. L. In *Burger's Medicinal Chemistry*, 4th ed.; Wiley-Interscience: New York, NY, 1980; Part 1, Chapter 9, pp 349–391.

afford the secondary ring-opened alcohol, **2bOH**, together with 2-methylcyclobutanols (reaction 15).[80] It will be obvious that cyclopropylmethyl cations cannot be intermediates in the oxidation

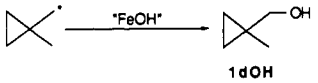


(15)

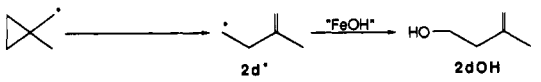
of **1aH** and the other methyl-substituted methylcyclopropanes since they would all very rapidly undergo rearrangements and yield alcohol products analogous to those shown in reaction 15. Thus, the hydroxylation of methylcyclopropanes by microsomal P-450 contrasts with the hydroxylation of at least one of these compounds by the P-450-like bacterial enzyme, *methane monooxygenase* (MMO), which has recently been shown to oxidize **1dH** by a *combination* of free-radical and cationic mechanisms.[81] The MMO oxidation of alkanes is thought to proceed via iron–oxygen activation/hydrogen abstraction steps similar to those in P-450 (reaction 16) but, with **1dH** as the substrate the reaction then appears to partition between a *direct hydroxyl (radical) rebound* (reactions 17 and 18) and an *indirect electron-transfer hyroxide (ion) rebound* mechanism (reactions 19 and 20).[81] The absence



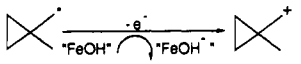
(16)



(81%) (17)



(6%) (18)



(19)

(13%) (20)

of both 1-methylcyclobutanol and **2dOH** in our P-450/**1dH** reaction indicates that the direct hydroxyl rebound is more efficient with P-450 than with MMO.

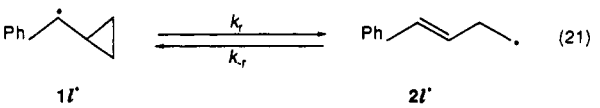
Returning to the well-behaved substrates **1bH** and **1cH**, we consider the most significant of all our experimental observations to be the fact that the microsomal oxidation of both of these substrates yield **2bOH/3bOH** product ratios which are equal within experimental error to the ratio of the rates of formation of the product radicals **2b•** and **3b•** from **1b•** and **1c•**, respectively, as determined by NRT in homogeneous solution at the same temperature.[48] Therefore, *for the two substrates **1bH** and **1cH** the corresponding carbon-centered radicals **1b•** and **1c•** which are formed by the P-450 undergo ring opening with exactly the same specificity as do the same radicals when they are genuinely free.* For this reason, we conclude that in the **1bH**/P-450 and **1cH**/P-450 systems the **1b•** and **1c•** radicals are unperturbed by their environment and, hence, that the four rate constants which can be calculated for oxygen rebound are valid measurements of this quantity. That is, for a small substrate which can yield a genuinely free radical unconstrained by its generation in the hydrophobic pocket of the enzyme, $k_{OH} \approx (1.7 \pm 0.2) \times 10^{10}$ s^{-1}.

Although the exclusive formation of *endo*-bicyclo[2.1.0]pentan-2-ol as the unrearranged alcohol from **4H** suggests that the motion of radical **4•** in the enzyme pocket is seriously restricted, the value calculated for k_{OH}, viz. 2.2×10^{10} s^{-1}, is in very satisfactory agreement with the values calculated from the two 1,2-dimethylcyclopropanes. It would therefore appear that the restricted motion of a radical does not necessarily influence the rate of oxygen rebound.

The small magnitude of the *intermolecular* primary deuterium kinetic isotope effect for bicyclo[2.1.0]pentane, viz., 1.3–1.7 (see Results), is consistent with earlier work using this substrate[42] and other substrates.[9-17]

Methylcyclopropane, **1aH**, and 1,1-dimethylcyclopropane, **1dH**, would appear to be well-behaved in that *if* radicals are formed in their oxidations by P-450 (as would appear to be almost certain) then the calculated lower limits on k_{OH} (viz. 1×10^{10} s^{-1}) are quite consistent with a "true" value for k_{OH} which has twice this magnitude.

Benzylcyclopropane, **1lH**, is also well-behaved in that the radical ring-opening reaction, **1l•** → **2l•**, is undoubtedly a slow process and is, in fact, much slower than the reverse ring-closing reaction, **2l•** → **1l•**.[82] Thus, we have demonstrated that for reaction 21



(21)

a claim[83] that $k_r = 2.7 \times 10^5$ s^{-1} and $k_{-r} \leq 3 \times 10^4$ s^{-1} at 22 °C is in error. By a combination of NRT of **1l•**[82,84] and tributyltin hydride reduction of 1,1-dideuterio-1-iodo-4-phenylbut-3-ene we have shown that reaction 21 is reversible at room temperature and that the equilibrium strongly favors the ring-closed radical, i.e., $k_{-r} \gg k_r$,[82,84] a conclusion which is consistent with thermochemical data.[85] Our tin hydride results yield $k_{-r} = 1.2 \times 10^7$ s^{-1} at 42 °C, and we can estimate from our failure to detect any ring-opened trialkylhydroxylamine in the NRT experiments that $k_r \leq 2 \times 10^5$ s^{-1}. The calculated lower limit on k_{OH} for **1lH** (viz., $>10^8$ s^{-1}) is therefore also consistent with the true values.

In short, benzylcyclopropane is not as useful a probe for radical intermediates in enzymatic reactions as has frequently been assumed.[86]

Oxygen Rebound for "Poorly Behaved" Clock Substrates. Two substrates are poorly behaved, 1,1,2,2-tetramethylcyclopropane, **1eH**, and hexamethylcyclopropane, **1fH**, because both yield radicals which undergo extremely rapid ring opening in homogeneous solution[48] and therefore both substrates would have been expected to yield substantial quantities of ring-opened alcohols. However, **1eh** gave *only a trace* of a ring-opened alcohol, and *no* ring-opened alcohol could be detected from **1fH**. These results allow us to put $k_{OH} = 2.5 \times 10^{11}$ s^{-1} for **1eH** and to put a lower limit on k_{OH} of 5×10^{11} s^{-1} for **1fH** (see Table I). Clearly, the results obtained with these two clock substrates are inconsistent with the message which is being transmitted by the three apparently "well-behaved" substrates, **1bH**, **1cH**, and **4H**.

The totally unexpected behavior of **1eH** and **1fH** could be attributed, in principle, to the operation of one or more of a numbers of factors. We have only been able to eliminate one of these possibilities by experiment. This was the possibility that **2f•**, the rearranged radical from **1fH**, reacted with the hydroxyferryl complex by disproportionation (reaction 7) rather than by oxygen rebound (reaction 8). This possibility is ruled out by our failure to detect 2,3,3,4-tetramethyl-1,4-pentadiene among the reaction products (see Results).

Other potential explanations for the failure of **1eH** and **1fH** to yield ring-opened alcohols include the following: (i) a change in the mechanism of hydroxylation, perhaps to a genuinely con-

(80) Caserio, M. C.; Graham, W. H.; Roberts, J. D. *Tetrahedron* **1960**, *11*, 171–182.

(81) Ruzicka, F.; Huang, D.-S.; Donnelly, M. I.; Frey, P. A. *Biochemistry* **1990**, *29*, 1696–1700.

(82) Bowry, V. W.; Lusztyk, J.; Ingold, K. U. *J. Chem. Soc., Chem. Commun.* **1990**, 923–925.

(83) Masnovi, J.; Samsel, E. G.; Bullock, R. M. *J. Chem. Soc., Chem. Commun.* **1989**, 1044–1045.

(84) See, also: Bowry, V. W. Ph.D. Dissertation, Australian National University, 1988. Beckwith, A. L. J.; Bowry, V. W. Unpublished results.

(85) See: Benson, S. W.; Cruickshank, F. R.; Golden, D. M.; Haugen, G. R.; O'Neal, H. E.; Rodgers, A. S.; Shaw, R.; Walsh, R. *Chem. Rev.* **1969**, *69*, 279–324. O'Neal, H. E.; Benson, S. W. In *Free Radicals*; Kochi, J. K.; Ed.; Wiley-Interscience: New York, NY, 1973; Vol. 2, Chapter 17. Rossi, M.; King, K. D.; Golden, D. M. *J. Am. Chem. Soc.* **1979**, *101*, 1223–1230.

(86) See, e.g.: Fitzpatrick, P. F.; Villafranca, J. J. *J. Am. Chem. Soc.* **1985**, *107*, 5022–5023.

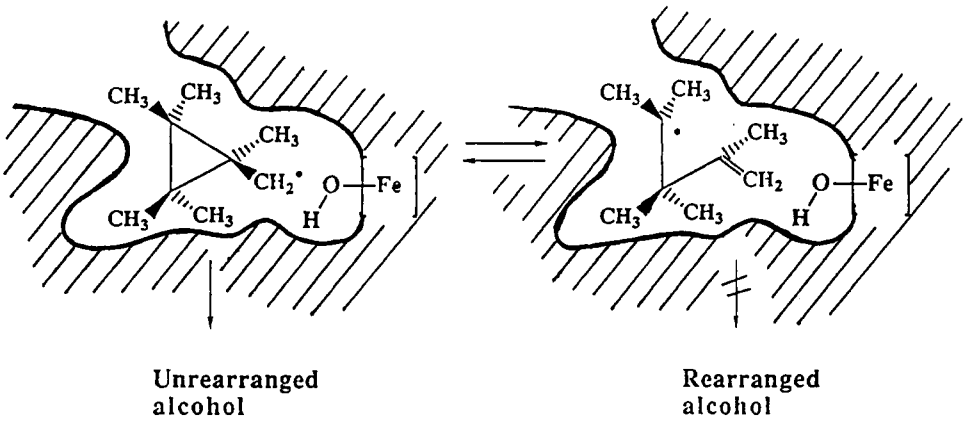


Figure 1. Artist's impression of **1f•** and **2f•** in the P-450 pocket.

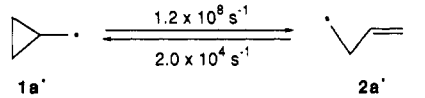
certed oxene insertion process; (ii) the occurrence of a radical mechanism in which there is a much faster oxygen rebound due, perhaps, to a much tighter binding of these two substrates to the enzyme's active site; (iii) a reduction in the (effective) rate of ring opening of **1e•** and **1f•**; and (iv) a reaction of the ring-opened radicals with the organic portion of the enzyme.

With regard to any of the above "explanations" it must not be forgotten that we are working with microsomes which contain a mixture of P-450 strains. Uninduced hepatic microsomes would appear to have the oxyferryl group at the bottom of a deep, narrow recess within the enzyme(s) since they show a reasonably high selectivity for the terminal methyl group during the hydroxylation of *n*-alkanes.[34,87] By way of contrast, the binding sites in hepatic microsomal P-450 which have been induced by phenobarbital and in purified P-450$_b$ must be fairly capacious since they can accommodate a large variety of competent substrates[31] and because they hydroxylate *n*-alkanes to a mixture of alkanols with a much stronger preference for the 2-alkanol than is the case for uninduced microsomes.[34]

Obviously, the problem posed by the possibility that the "larger" clock substrates, **1eH** and **1fH**, are oxidized by a different strain (or strains) of P-450 from that (or those) which oxidize the "smaller" clock substrates, **1aH–1dH** and **4H**, can only be resolved by experiments using the same substrates and one or more purified strains of P-450. While such definitive experiments are planned, we believe that simpler, and hence more probable, explanations for the unusual behavior of the larger substrates reside in possibilities (iii) and (iv) given above and that different strains of P-450 are not involved.

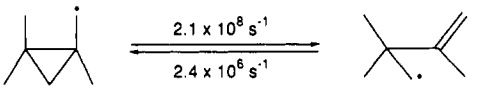
The *effective* rate of ring opening of the radicals **1e•** and **1f•** derived from the larger substrates might be reduced because of steric constraints imposed by the hydrophobic pocket in the enzyme in which they are generated, such effects being absent for the radicals derived from the smaller clock substrates. That substrate size *can* have a profound effect on the overall reaction is forcibly indicated by the failure of dispiro[2.2.2.2]decane, **1kH**, to undergo any detectable oxidation by P-450. The failure of **1kH** to react is *not* due to the low reactivity of the hydrogens on the cyclohexane ring toward abstraction by oxygen-centered radicals.[48] Indeed, toward *tert*-butoxyl radicals and on a per hydrogen basis, the secondary cyclohexyl hydrogens of **1kH** are 5 times as reactive as the secondary cyclobutyl hydrogens of **4H** and are 21 and 15 times as reactive as the primary hydrogens of **1eH** and **1aH**, respectively.[48]

One can imagine a steric effect on the effective rate of ring-opening operating in one of two ways (or, indeed, more probably in both ways): first, a direct retardation of the rate of the ring-opening reaction and second, a direct acceleration of the reverse, ring-closing reaction. With regard to the first of these two possibilities, we note that although it has been shown[88] that the ring opening of the cyclopropylmethyl radical, **1a•** → **2a•**, is not *prevented* by confinement in a rigid, 5.5-Å diameter channel in a crystal of urea, the *rate* of the ring opening, which was not measured, may well have been retarded. With regard to the second possibility, it must not be forgotten that cyclopropylcarbinyl radical ring-opening reactions are reversible.[89,90] Thus, for the 3-butenyl radical, **2a•**, the rate constant for ring closure to cyclopropylmethyl, **1a•**, has a value of 2.0×10^4 s^{-1} at 37 °C,[91] i.e., $K = 6 \times 10^3$ (reaction 22). For the methyl-substituted cyclopropylmethyl



(22)

radicals ring opening is accelerated (see Table I), but ring closing can be accelerated to an even greater extent. For example,[48] for the 1,2,2-trimethylcyclopropylmethyl radical, **1e•**, ring opening to **3e•** is accelerated by less than a factor of 2, but ring closing is accelerated more than 100-fold, $K = 88$ (reaction 23). Of



(23)

course, the corresponding tertiary radical, **2e•**, which is the major ring-opened radical, would be expected to recyclize to **1e•** at a much slower rate than the primary radical, **3e•**. Nevertheless, the enzyme's hydrophobic pocket may be sufficiently "restrictive" that it not only hinders the ring opening of **1e•** and **1f•** but also accelerates the ring closure of **2e•**, **3e•**, and **2f•** (see Figure 1). Furthermore, the pocket might be able to retard the "turnaround" of the ring-opened radicals thus making it more difficult for them to "present" the new radical center to the FeOH species. In addition, there is the possibility that the tertiary alkyl radicals, **2e•** and **3e•**, are simply too bulky to be readily hydroxylated even when they are correctly positioned with respect to the FeOH moiety.

With regard to possibility (iv), if the bulky, ring-opened radicals **2e•**, **3e•**, and **2f•** abstract hydrogen from, or add to, the organic portion of the enzyme, this would presumably deactivate the enzyme. The yields of **1eOH** and **1fOH** did not appear to be unusually low. However, it will take a great deal of painstaking effort to measure partition numbers for a sufficient variety of alkanes to decide whether **1eH** and **1fH** are particularly effective as "suicide" substrates toward the enzyme. As noted previously, such experiments are currently in progress.

(87) See, also: Bernhard, K.; Gloor, U.; Scheitlin, E. *Helv. Chim. Acta* **1952**, *35*, 1908–1913. Ichihara, K.; Kusunose, E.; Kusunose, M. *Biochim. Biophys. Acta* **1969**, *176*, 713–719.

(88) Casal, H. L.; Griller, D.; Kolt, R. J.; Hartstock, F. W.; Northcott, D. M.; Park, J. M.; Wayner, D. D. M. *J. Phys. Chem.* **1989**, *93*, 1666–1670.

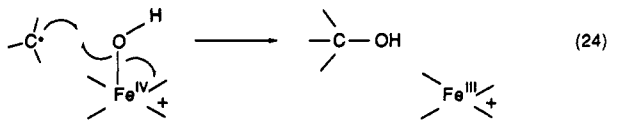
(89) Wilt, J. In *Free Radicals*; Kochi, J. K., Ed.; Wiley-Interscience: New York, NY, 1973; Vol. 1, Chapter 8.

(90) Beckwith, A. L. J.; Ingold, K. U. In *Rearrangements in Ground and Excited States*; de Mayo, P.; Ed.; Academic: New York, NY, 1980; Vol. 1, Essay 4.

(91) Effio, A.; Griller, D.; Ingold, K. U.; Beckwith, A. L. J.; Serelis, A. K. *J. Am. Chem. Soc.* **1980**, *102*, 1734–1736.

Mechanism of Oxygen Rebound. The detailed mechanism of hydroxylation of carbon-centered radicals (reaction 3) has generally been either ignored or considered to be a combination of the carbon-centered radical with a hydroxyl radical.[92] The latter mechanism implies that the iron–oxygen bond in the hydroxyferryl species must undergo thermal cleavage before hydroxylation can occur. This is, of course, improbable both on energetic grounds and because an enzyme which generated a free hydroxyl radical would not survive for long. Furthermore, the reaction of an alkyl radical with a free hydroxyl radical would be expected to yield disproportionation products, i.e., alkene and water as well as the alcohol combination product. In the case of hexamethylcyclopropane we have shown that olefin is not produced, and hence we rule out the production of a free hydroxyl radical.

The actual hydroxylation step most probably involves a bimolecular homolytic substitution[94] (S_H2) at oxygen of carbon for iron,[95] i.e., reaction 24. We cannot rule out, but see no reason



(24)

to invoke, a direct interaction between the carbon-centered radical and the iron atom to form an Fe^V species; i.e., we see no reason to invoke an intermediate with a carbon–iron bond, particularly in such a "crowded" local environment.

Finally, in all cases where there is no clear evidence for the intermediate formation of carbon-centered radicals, it must not be forgotten that an oxene insertion mechanism may be in operation.

Acknowledgment. We thank Dr. D. O. Foster for preparation of the P-450 microsomes, D. A. Lindsay for his valuable technical assistance, E. Lusztyk for a second, extremely careful examination of the products formed from the P-450-catalyzed oxidation of **1eH**, and the Association for International Cancer Research and the National Foundation for Cancer Research for partial support of this work. We also thank Prof. J. Warkentin for generously providing a sample of 3-methoxy-3,5,5-trimethyl-4-oxa-1-pyrazoline and two referees for some very useful suggestions.

Registry No. 1aH, 594-11-6; **1aOH**, 2516-33-8; **1a•**, 2154-76-9; **1bH**, 2402-06-4; **1bOH**, 21003-36-1; **1b•**, 62131-99-1; **1cH**, 930-18-7; **1cOH**, 21003-35-0; **1c•**, 62131-98-0; **1dH**, 1630-94-0; **1dOH**, 2746-14-7; **1d•**, 24389-71-7; **1eH**, 4127-47-3; **1eOH**, 133753-26-1; **1e•**, 133753-28-3; **1fH**, 2570-81-2; **1fOH**, 133753-27-2; **1f•**, 133753-29-4; **1kH**, 24518-94-3; **1lH**, 1667-00-1; **1lOH**, 1007-03-0; **1l•**, 126281-30-9; **2a•**, 2154-62-3; **2b•**, 51685-66-6; **2bOH**, 24389-75-1; **2eOH**, 19781-53-4; **2e•**, 50517-76-5; **2fOH**, 4819-92-5; **2f•**, 133753-31-8; **2lOH**, 937-58-6; **2l•**, 133753-32-9; **3bOH**, 4516-90-9; **3b•**, 52898-42-7; **3eOH**, 3329-43-9; **3e•**, 133753-30-7; **4H**, 185-94-4; **$d_2$4H**, 51794-28-6; **4OH**, 24461-57-2; **4•**, 84592-00-7; **5OH**, 14320-38-8; **5•**, 14461-09-7; cytochrome P-450, 9035-51-2; monooxygenase, 9038-14-6; 2-butyn-1-ol, 764-01-2; ethyl dimethylacetoacetate, 597-04-6; ethyl 2,2,3-trimethyl-3-butenoate, 35293-39-1; methyl 1,2,2-trimethylcyclopropanecarboxylate, 20459-94-3; 3-methoxy-3,5,5-trimethyl-4-oxa-1-pyrazoline, 77879-49-3; 4-phenyl-3-butenoic acid, 2243-53-0.

(92) For example,[93] "attack of this iron–oxo species on the substrate molecule through hydrogen abstraction followed by radical recombination to generate the alcohol product".

(93) Murray, R. I.; Fisher, M. T.; Debrunner, P. G.; Sligar, S. G. *Topics in Molecular and Structural Biology* **1985**, *6*, 157-206. See: p 191.

(94) Roberts, B. P.; Ingold, K. U. *Free-Radical Substitution Reactions*; Wiley-Interscience: New York, 1971.

(95) Champion, P. M. *J. Am. Chem. Soc.* **1989**, *111*, 3433-3434.

Vases and Kites as Cavitands[1]

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Abstract: The syntheses, characterizations, and substituent effects on the vase vs kite conformations of **1–17** are described. These compounds are assembled by two-step syntheses from resorcinol (or 2-substituted derivatives) and aldehydes to form octols **18–26** in high yields, followed by 4-fold bridging reactions with quinoxalines **27–29** or pyrazine **30**. In the crystal structure of **3**·2CH_2Cl_2, one CH_2Cl_2 is enclosed in the vase cavity, while a second CH_2Cl_2 is found surrounded by the four $(CH_2)_4Cl$ groups. When the 2-position of resorcinol is hydrogen, only the vase form of the cavitands exists at 25 °C or higher when quinoxaline bridged, as in **1–7**, and at all available temperatures when pyrazine bridged, as in **13**. The R and B groups of **1–7** can be varied to control solubility and cavity size without greatly affecting the vase–kite structures. When the 2-position of resorcinol is occupied by a methyl, an ethyl, or a bromine, as in **14–17**, only the kite conformation is observed at all available temperatures. When the 2-position is hydrogen and the system is quinoxaline, only the kite conformer is observed at temperatures below −50 °C. When the 2-position is CH_3, the kite conformer equilibrates with its dimer. When the 2-position is CH_3CH_2, as in **17**, the kite conformer does not form a dimer. The kite C_{2v} structures under pseudorotation and also dimerize when they contain 2-methylresorcinyl groups to give dimers of D_{2d} symmetry. In some systems, these processes could be differentiated by use of variable-temperature ^1H NMR spectra.

In an earlier paper,[2] we described the preparation and equilibration of the vase (four quinoxaline flaps axial, or aaaa conformation) and kite (four quinoxaline flaps equatorial, or eeee conformation) structures for **1**. Compound **1** was reported to form crystalline solvates that were stable to moderate heat and vacuum, which suggested the solvent molecules occupied the sizable cavity as guests of **1**. In a later paper, Vincenti, Dalcanale, Soncini, and Guglielmetti found that an analogue of **1** (R = C_6H_{13}) bound guest molecules strongly in the gas phase.[3] Our paper reports the following: (1) the syntheses of cavitands **1–17** and **31** and octols **21** and **25**; (2) the crystal structure of the vase form of **3**·2CH_2Cl_2; and (3) the results of an investigation of the effects of substituents

(1) (a) We warmly thank the National Science Foundation for Grant CHE 88-02800, which supported this work. (b) Host–Guest Complexation. 57.

(2) Moran, J. R.; Karbach, S.; Cram, D. J. *J. Am. Chem. Soc.* **1982**, *104*, 5826-5828.

(3) Vincenti, M.; Dalcanale, E.; Soncini, P.; Guglielmetti, G. *J. Am. Chem. Soc.* **1990**, *112*, 445-447.

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