Hydroxylation by Cytochrome P-450 and Metalloporphyrin Models. Evidence for Allylic Rearrangement

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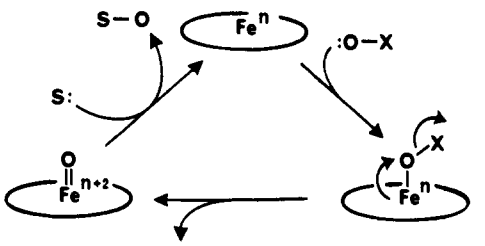
Abstract: The allylic hydroxylation of 3,3,6,6-tetradeuteriocyclohexene, methylenecyclohexane, and β-pinene has been examined with phenobarbital-induced liver microsomal cytochrome P-450 ($P\text{-}450_{LM2}$) and with iron porphyrin and chromium porphyrin model systems. Aerobic and peroxide dependent enzymic regimes were investigated with purified $P\text{-}450_{LM2}$ and with microsomal suspensions. Epoixidation and allylic hydroxylation were the primary reactions with all substrates. With 3,3,6,6-tetradeuteriocyclohexene, the major hydroxylation product (60–80%) was the result of hydroxylation at the deuterated allylic site. In all cases, a significant amount (20–40%) of hydroxylation occurred with allylic rearrangement. The iron porphyrin/iodosylbenzene model system also showed preferential hydroxylation of the deuterated allylic site (70%) with significant allylic rearrangement (30%). By contrast, the chromium porphyrin/iodosylbenzene model system showed complete scrambling of the allylic system. Extensive rearrangement accompanied the hydroxylation of methylenecyclohexane and β-pinene by both the enzymic and metalloporphyrin systems whereas the selenium dioxide oxidation of these substrates gave selective allylic hydroxylation without rearrangement. A mechanism is suggested for allylic hydroxylation by cytochrome P-450 and by the metalloporphyrin model systems involving initial hydrogen atom abstraction from the allylic site and geminate, cage recombination of the incipient, allylic free radical.

The details of the mechanism of action of cytochrome P-450 have been elusive due to the instability of the intermediates in the catalytic cycle and the lack of simple model systems with similar reactivity. An attractive approach to the elucidation of the chemical nature of the oxygen-transfer event has been the design of substrate molecules that have the potential to reveal the mechanism of oxygen transfer and, hence, the nature of the active species via diagnostic rearrangement pathways. For the hydroxylation of aliphatic substrates by cytochrome P-450, we[1] and others[2] have shown that hydrogen removal is accompanied by a very large isotope effect (k_H/k_D = 10–12) and that the hydroxylation event occurs with significant loss of stereochemistry at the oxidized carbon.[1,3] Taken together, these results support a stepwise, homolytic path for hydroxylation involving an initial hydrogen abstraction by an oxy radical species. Two types of oxy radicals may be reasonably considered: an oxyferryl species formally equivalent to iron(V)[4] or an organic radical species derived from the protein.[5] Model studies in our laboratory with synthetic porphyrin complexes have shown that a reactive iron(IV) porphyrin cation radical can be formed at low temperatures which is capable of hydroxylation and epoxidation of typical hydrocarbons.[6] This chemical precedent and its similarity to HRP compound I[7] support the oxygen rebound path for oxygen transfer[8] (Scheme I) in which a peroxyferric heme (**1**) decomposes by heterolytic cleavage of the O–O bond to give the reactive oxyferryl species (**2**).

To further probe the hydroxylation event and to establish similarities and differences between the enzymic and model reactions, we have examined the oxidation of several olefinic substrates with purified and microsomal cytochrome P-450 and synthetic porphyrin/iodosylarene model systems. Preliminary studies had indicated that significant allylic rearrangement accompanied the hydroxylation of 1,2-dideuteriocyclohexene with

Scheme I



cytochrome P-450 and with an iodosylbenzene/chloro(tetraphenylporphyrinato)iron(III) [Fe(TPP)Cl] model system.[9] We describe here a more complete investigation of allylic hydroxylation of cytochrome P-450 and the model metalloporphyrin systems in which allylic rearrangement has been found to be a general result.

Results

Oxidation of 3,3,6,6-Tetradeuteriocyclohexene. As we have shown elsewhere,[10] cyclohexene is oxidized by a fully reconstituted system containing cytochrome $P\text{-}450_{LM2}$, cytochrome P-450 reductase, NADPH, and oxygen to give cyclohexene oxide and 2-cyclohexen-1-ol in approximately equivalent amounts. Similar results were obtained with cumene hydroperoxide (CHP) and iodosylbenzene dependent systems. For 3,3,6,6-tetradeuteriocyclohexene, the epoxide/enol ratio was 4.7 for the fully reconstituted system. The direct determination of deuterium position was not possible for the product enol or its trimethylsilyl ether due to the facile allylic rearrangement of the mass spectral molecular ions.[11] The oxidation of 2-cyclohexen-1-ol to cyclohexenone with manganese dioxide in ether has been shown to be complete within 30 min at room temperature.[9] The intense parent and parent minus ethylene peaks in the mass spectrum of cyclohexenone allowed accurate measurement of the deuterium content after appropriate correction for the ^{13}C content of the ions was made since these strong peaks are uncomplicated by proton loss. The oxidation of 1-deuterio-2-cyclohexen-1-ol with manganese dioxide under these conditions afforded cyclohexenone with 100% loss of allylic deuterium label. Thus, no allylic scrambling

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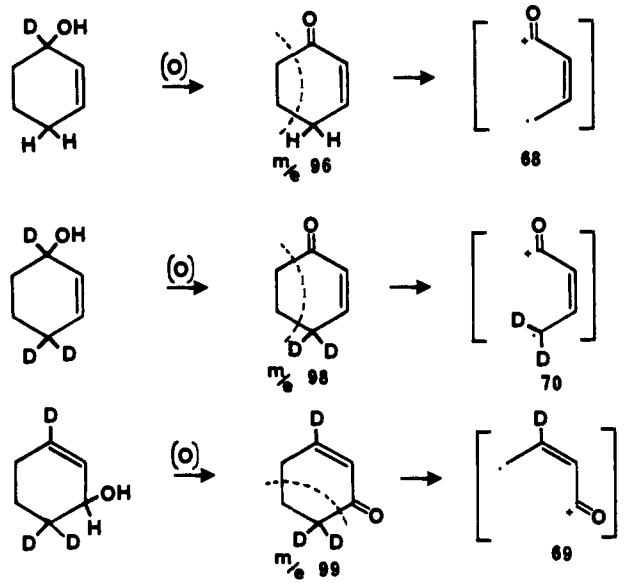
Table I. Mass Spectral Data for Deuterated Cyclohexenones

	m/e						m/e							
conditions	68	69	70	71	% d_2	% d_3	96	97	98	99	100	% d_2	% d_3	% retn
P-450/O_2	15.3	64.6	100	12.4	61	39	0.71	2.76	27.8	20.2	0.27	60	40	33.5
	10.7	47.8	100	8.51	67	33	0.40	0.78	25.3	8.54	1.41	79	21	
P-450/CHP	13.7	39.5	100	8.00	72	28	0.24	0.98	32.3	8.59	1.41	80	20	50.5
	13.6	38.5	100	8.67	72	28		0.40	30.7	11.0	0.26	77	23	
PB microsomes/O_2	13.4	26.6	100	6.49	79	21	1.66	0.84	24.9	7.34	0.61	81	19	
					(75)	(25)[c]						(77)	(23)[c]	56
PB microsomes/CHP	22.4	32.8	100	8.57	76	24	3.72	0.55	27.3	8.21	0.78	81	19	54.5
	9.56	37.0	100	6.43	73	27			20.5	6.70	0.56	79	21	
Fe(TPP)Cl/C_6H_5IO	12.7	51.8	100	10.2	66	34	0.55	0.75	25.3	12.4	1.44	70	30	37.5
		48.8	100	9.2	67	33			17.5	8.02		72	28	
	14.0[a]	100		88.5	44.2	46	54		2.91	24.2	26.6	5.26	49	51	
Cr(TPP)Cl/C_6H_5IO	12.2	100		93.0	15.4	47	53	0.33	0.46	25.7	28.6	0.75	49	51	-4
	5.96[a]	100		38.6	15.5	25	75	0.22	0.39	7.94	24.2	1.30	25	75	
SeO_2	15.4	20.3	100	6.79	87	13	0.34	0.80	37.3	8.28	0.35	84	16	70
P-450/CHP[b]		32.8	100					6.7	21.4			24	76	52
Fe(TPP)Cl/C_6H_5IO[b]		56.6	100					10.0	18.3			37	63	26
Cr(TPP)Cl/C_6H_5IO[b]		100		95.2				21.8	21.6			52	48	-4

[a] Cyclohexenone present prior to MnO_2 oxidation. [b] For the oxidation of 1,2-dideuteriocyclohexene; cf. ref 9. [c] An independent determination.

Scheme II



resulted from this oxidation, and the position of the hydroxyl group in 2-cyclohexen-1-ol relative to the deuterium labels could be related directly to the deuterium content of the cyclohexenone from the MnO_2 oxidation (Scheme II). Complete oxidation of the cyclohexenol samples assured that the results would be unaffected by any intermolecular isotope effects for the manganese dioxide oxidation. Mass spectral data for the cyclohexenone obtained from the manganese dioxide oxidation of the hydroxylation products of 3,3,6,6-tetradeuteriocyclohexene by the fully reconstituted cytochrome P-450$_{LM2}$ system, the peroxide-dependent P-450 system, and hepatic microsomes containing cytochrome P-450 are presented in Table I. The deuterium content of the product cyclohexenone obtained from each of these regimes was calculated independently from the parent and parent minus ethylene regions of the mass spectrum. The close correlation of the parent and fragment ion results in reassuring evidence that the enone fragmentation occurs as indicated in Scheme II. The relative prominence of peaks at m/e 98 and 70 indicates that the major hydroxylation path for purified and microsomal P-450 (60–80%) was hydroxylation of the deuterated allylic methylene group of 3,3,6,6-tetradeuteriocyclohexene. The peaks at m/e 99 and 69, however, show that in each case significant amounts (20–40%) of hydroxylation with allylic rearrangement had occurred.

The oxidation of cyclohexene by iodosylbenzene catalyzed by Fe(TPP)Cl in methylene chloride produced a mixture of cyclohexene oxide and cyclohexenol in a ratio of 3.7:1 (70% based on iodosylbenzene).[12] When oxygen was rigorously excluded from the reaction mixture the amount of cyclohexenone produced, though detectable, was negligible compared to the amount of cyclohexenol. With cyclohexene-d_4, the ratio of epoxide to enol was 14.7 (k_H/k_D = 4) and the amount of cyclohexenone produced was 0.5–2% of that of the enol. The d_3 and d_2 content of this cyclohexenone was 51% and 49%, respectively. By contrast, the cyclohexenone produced upon manganese dioxide oxidation of the cyclohexene showed a deuterium distribution pattern indicative of preferential (70%) hydroxylation of the deuterated allylic positions (Table I). As with the cytochrome P-450 oxidations, a significant amount of cyclohexenone (30%) derived from cyclohexenol which had undergone allylic rearrangement during hydroxylation.

The oxidation of 3,3,6,6-tetradeuteriocyclohexene with Cr(TPP)Cl/iodosylbenzene[13] produced cyclohexenol, small amounts of cyclohexene oxide, and a significant amount of cyclohexenone (37% based on total allyl oxidation) even in the rigorous absence of oxygen. The deuterium content of the cyclohexenone initially formed was 77% d_3 and 23% d_2. The oxidation of the cyclohexenol–cyclohexenone mixture produced in the catalytic oxidation by Cr(TPP)Cl with manganese dioxide in ether gave samples of cyclohexenone with a nearly statistical distribution of deuterium (Table I). In a separate experiment Cr(TPP)Cl was shown to catalyze the oxidation of 2-cyclohexen-1-ol to cyclohexenone. Thus, the distribution of deuterium in the initially formed portion of cyclohexenone must be the result of an isotope effect (k_H/k_D = 4.0) for the oxidation of the allylic alcohol. The change to a statistical distribution of deuterium in the cyclohexenone upon manganese dioxide oxidation of the remaining cyclohexenol indicates that the initial allylic hydroxylation catalyzed by Cr(TPP)Cl had occurred with complete allylic scrambling and that the pool of 1-proteo-2-cyclohexen-1-ol had been depleted by the subsequent oxidation of cyclohexenone.

Selenium dioxide has found wide application as a reagent for the selective allylic hydroxylation of olefins.[14] The mechanism of this process has been shown to involve two concerted processes: an "ene" reaction to give an allyl selenic acid and subsequent [2.3] sigmatropic rearrangement of this intermediate to introduce the hydroxyl function at the original allylic position.[15] The oxidation of 3,3,6,6-tetradeuteriocyclohexene with selenium dioxide in 1-

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Table II. Oxidation of Methylenecyclohexane

conditions	products 	 3	 4	retn/rearr
P-450/O_2	19[a]	2.8	2.37	54/46
P-450/CHP	1.0	3.58	5.14	41/59
P-450/*t*-BuOOH	1.36[b]	2.77	3.53	44/56
PB microsomes/ *t*-BuOOH	tr[c]	2.33	3.07	43/57
PB microsomes/ NADPH/O_2	tr[c]	0.605	0.431	58/42
Fe(TPP)Cl/ $Me_3C_6H_2IO$[d]	528[e]	22[h]	6.7[h]	67/33
Cr(TPP)Cl/ $Me_3C_6H_2IO$	15.3[f,g]	3.24[i]	1.59[i]	43/57
SeO_2		92[j]	3[j]	95/5

[a] 24 nmol of product/nmol of P-450. [b] 7.66 nmol of product/nmol of P-450. [c] The epoxide was unstable to PB microsomes. [d] Iodosylmesitylene, 4.5 μmol. [e] Product yield 38% based on $Me_3C_6H_2IO$; 2.97 nmol/nmol Fe(TPP)Cl. [f] Small amounts of 1-chloromethylcyclohexene were also detected. [g] Product yield based on iodosylmesitylene; 0.75 nmol/nmol Cr(TPP)Cl. [h] Ketone 1.0, aldehyde 4.7. [i] Ketone 3.24, aldehyde 4.12. [j] Ketone 3, aldehyde 2.

Table III. Oxidation of β-Pinene

conditions						retn/ rearr
P-450/O_2	36.5[a,b]	15.8[c]	6.45	15.2	1	57/43
P-450/C_6H_5IO	55[d]	63[c]	10.3	12	1	85/15
PB microsomes/ C_6H_5IO	1[e]	32[c]	8	15	17.7	55/45
PB microsomes/ NADPH/O_2	*e*	59[c]		41		59/41
Fe(TPP)Cl/ C_6H_5IO	38.2[f,g]	3.46[c]	12.35	5.1	1	72/38
Cr(TPP)Cl/ C_6H_5IO	0.75[h,i]	2.5	1.0	1.6	1.7	52/48
SeO_2		30	2	0.30	1	96/4

[a] 26 nmol of product/nmol of P-450. [b] Cis/trans = 1.62. [c] Syn alcohol was absent. [d] 6.44 nmol of product/nmol of P-450. [e] This product was unstable to PB microsomes. [f] Product yield 61% based on iodosylbenzene; 4.84 nmol of product/nmol of Fe(TPP)Cl. [g] Cis/trans = 1.32. [h] Product yield 54% based on iodosylbenzene; 4.3 nmol of product/nmol of Cr(TPP)Cl. [i] Cis/trans = 1.47.

butanol/pyridine and subsequent manganese dioxide oxidation gave cyclohexenone which was largely (85%) unrearranged (Table I). Independent studies by Stevenson[16] and by Sharpless[17] have shown that significant homolysis and solvolysis of the intermediate selenic acid occur in this reaction, particularly for endocyclic olefins. The 15% allylic rearrangement observed here is consistent with these reports.

Oxidation of Methylenecyclohexane and β-Pinene. The products of the oxidation of methylenecyclohexane by the reconstituted P-450 system were found to be the corresponding epoxide and two allylic alcohols (**3** and **4**) in a ratio of 8:1.2:1. For the cumeme hydroperoxide dependent system the ratio was 1:3.58:5.14 due to a decrease in the absolute amount of epoxide produced. Similar mixtures of products, including rearranged allylic alcohols but with more noticeable carbonyl products, were observed for the oxidation of β-pinene by the enzymic and porphyrin-catalyzed processes. No epoxides were detected in the microsomal oxidations. Controls indicated that while cyclohexene oxide was stable to the microsomal suspensions, the epoxides of methylenecyclohexene and β-pinene were not. Epoxide hydrase is a known constituent of these microsomal fractions.[5b] Data for the oxidation of methylenecyclohexane and β-pinene are presented in Tables II and III. The oxidation of methylenecyclohexane and β-pinene with selenium dioxide gave very clean allylic hydroxylation. Since the products of selenium dioxide oxidation were subjected to the identical workup and GC assay conditions as those of the enzymic and porphyrin-catalyzed reactions and since product ratios were unaffected by exposure to these latter reaction conditions, it can be concluded that none of the observed rearrangements occurred after the hydroxylation event.

Discussion

The results indicate that the allylic hydroxylation of cyclohexene mediated by cytochrome P-450 occurs predominantly (60–80%) without rearrangement of the double bond. Further, the results for aerobic oxidations by the reconstituted and microsomal systems were qualitatively similar to the cumene hydroperoxide dependent oxidations. The amount of rearranged alcohol cannot be due to rearrangements occurring subsequent to hydroxylation since 1-deuterio-3-cyclohexen-1-ol was stable and not subject to rearrangement under the reaction conditions and no rearrangement occurred during the manganese dioxide oxidation. Unreacted cyclohexene-d_4 was also recovered unchanged. The analysis of the results is insulated from possible effects of partial deuteration. As a result of the large isotope effect, the contribution of the 2% cyclohexene-d_3 will be amplified. However, *hydrogen removal* from cyclohexene-d_3 will produce intermediates with the same deuterium content and distribution as deuterium removal from cyclohexene-d_4. Further, the similarity of the results described here to those we have reported for 1,2-dideuteriocyclohexene show that the position of preferred hydroxylation is not the result of an isotope effect. Extensive allylic rearrangement also accompanied the hydroxylation of methylenecyclohexane and β-pinene. Thus, the mechanism of allylic hydroxylation by P-450 must accommodate the observed oxygen transfer with scrambling of the allylic positions. Significantly, the iron porphyrin/iodosylbenzene model system showed a similar degree of allylic scrambling and a memory effect was evident for the hydroxylation of cyclohexene.

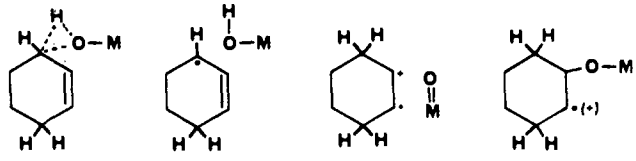
The possibility that the nonspecific portion of the reaction occurs as the result of autoxidation or free-radical chain reactions in solution can be discounted. Such reactions of cyclohexene always produce cyclohexenone as a primary product. Since cyclohexenone was not observed as a product of cyclohexene oxidation by the enzyme and is only a trace product in the iron porphyrin catalyzed oxidation in the absence of oxygen, such a free-radical chain process appears unlikely.[18] The small amounts of cyclohexenone produced in the Fe(TPP)Cl-catalyzed oxidation of cyclohexene had a statistical distribution of deuterium. This is the result expected for an autoxidation involving a discrete allylic radical intermediate. Since the cyclohexenone produced in this way was always less than 2% of the total allylic oxidation, the amount of autoxidation of the iron porphyrin system must also be negligible. That no oxidation had occurred during the workup and assay of the enzymic oxidations was confirmed by the addition of cyclohexene to a 3,3,6,6-tetradeuteriocyclohexene reaction mixture immediately after quenching the reaction. Upon MnO_2 oxidation and GC–MS assay, only trace amounts of cyclohexenone-d_0 were observed.

Considerable evidence has accumulated in support of an oxo-iron(IV) porphyrin cation radical (**5**) as the reactive species responsible for epoxidation and hydroxylation by the iron porphyrin system. Visible and NMR spectra of **5** are indicative of an A_{2u} state for the porphyrin monoanion.[6] The temperature dependence of the Mössbauer spectrum of **5** is consistent with a low-spin iron(IV) strongly and isotropically coupled to the porphyrin spin.[19]

(16) Stevenson, L. M.; Speth, D. R. *J. Org. Chem.* **1979**, *44*, 4683–4489.
(17) Warpehoski, M. A.; Chabaud, B.; Sharpless, K. B. *J. Org. Chem.* **1982**, *47*, 2897–2900.
(18) See ref 24a.
(19) Boso, B.; Lang, G.; McMurry, T. J.; Groves, J. T. *J. Chem. Phys.* **1983**, *79*, 1122–1126.

Further, EXAFS data show a feature at 1.6 Å which can only be due to the iron–oxo group.[20] A molecular orbital description of **5** has two half-filled, $d\pi$–$p\pi$ antibonding orbitals associated with the iron–oxo group. Accordingly, the oxygen in **5** is expected to have considerable oxy radical character.

Several limiting mechanisms for allylic hydroxylation by such an oxoiron(IV) porphyrin cation radical are (i) concerted oxygen

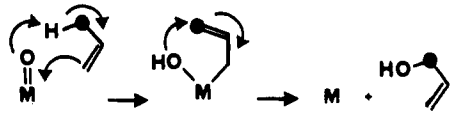


i ii iii iv

insertion into the allylic C–H bond, (ii) allylic hydrogen atom abstraction and geminate radical recombination, (iii) electron transfer from the olefin to give a π-cation radical that subsequently loses an allylic proton or hydrogen atom, (iv) addition of metal-bound oxygen to the double bond followed by elimination of hydrogen.

While mechanism iv has the attraction of a possible common intermediate for hydroxylation and epoxidation, this path predicts that allylic hydroxylation should occur with rearrangement of the double bond,[21] and it does not provide a path for hydroxylation without rearrangement. A preference for allylic hydroxylation without rearrangement in cyclohexene is not easily explained by mechanism iii either since the oxidant must interact initially with the double bond. Loss of hydrogen from an intermediate π-cation radical would not be expected to occur with a large isotope effect for allylic hydroxylation.[22] We have shown that the allylic hydroxylation of cyclohexene by cytochrome P-450[10] and the iron porphyrin system occurs with a substantial hydrogen isotope effect (k_H/k_D = 4–5). Accordingly, while path iii is a conceivable route for epoxidation,[23] it is unlikely for hydroxylation. A concerted, oxygen insertion mechanism (path i) would give predominant hydroxylation of the deuterated allylic site, as observed with 3,3,6,6-tetradeuteriocyclohexene, but such a mechanism cannot explain the significant amounts of allylic rearrangement that accompany hydroxylation in every case. Indeed, for methylenecyclohexene, the rearrangement path predominates over insertion.

Sequential concerted steps via an organometallic intermediate (**6**) in analogy with the mechanism of allylic hydroxylation by

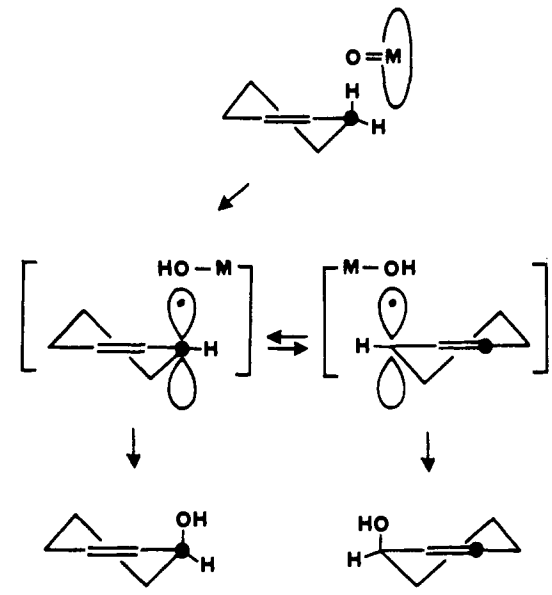


6

selenium dioxide[15-17] could accommodate the observed results if the subsequent reductive elimination of **6** proceeded in a cyclic, five-membered ring fashion. This type of process would be unique for allylic substrates, however, and could not explain the epimerization that occurs upon hydroxylation of isolated methylene groups.[1,3]

A mechanism involving allylic hydrogen atom abstraction and cage recombination of the incipient carbon radical (path ii) is consistent with all of the observed results (Scheme III). The degree of allylic scrambling in such a process would be intimately related to the lifetime of the caged radical species. Three factors

Scheme III



affecting the expected lifetime of the caged radical are the stability of the carbon radical, the steric accessibility of the carbon radical center, and the reactivity of the metal oxide toward carbon radicals.[24] We have reported that the hydroxylation of norbornane by cytochrome P-450$_{LM2}$ occurs with partial epimerization of the methylene group during hydroxylation.[1] Likewise, Sligar has demonstrated that the 5-exo-hydroxylation of 5-deuteriocamphor by P450$_{cam}$ occurs with removal of either the exo or endo hydrogen.[3] Hydroxylation with partial epimerization is consistent with a radical cage process.[25] The partial epimerization that accompanies aliphatic hydroxylation by simple transition-metal oxo reagents such as chromates has also been attributed to a radical cage process.[26] The greater stability of allylic radicals and the modest molecular motion required to achieve allylic rearrangement in a cage process would suggest a greater degree of rearrangement for allylic hydroxylation over the hydroxylation of an unactivated methylene group. The extensive rearrangement observed with the two exocyclic olefins by cytochrome P-450 supports this view. Further, it is expected on these grounds that hydroxylation of a methyl group should show less loss of stereochemistry since the primary radical is less stable than the allylic or secondary cases and the terminal methylene radical would have smaller steric constraints for recombination. In such a case an intimate cage recombination path may be indistinguishable from insertion.[27,28]

The statistical distribution of deuterium in the hydroxylation product of 3,3,6,6-tetradeuteriocyclohexene catalyzed by Cr(TPP)Cl also supports the caged radical scheme. The reactive species in the chromium catalyzed process has been shown to be an oxochromium(V) complex (**7**).[13] Hydrogen atom abstraction from cyclohexene by **7** would produce a hydroxochromium(IV) complex (**8**) and a cyclohexenyl radical. Deprotonation of **8** would give a stable oxochromium(IV) species (**9**) which we have shown to be very unreactive toward hydrocarbons.[29] Thus, slow collapse

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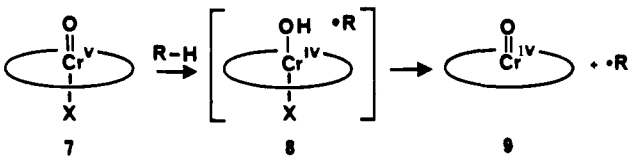

7 8 9

of **8** or diffusion of the allylic radical from the metalloporphyrin site would allow time for complete scrambling of the deuterium label. Diffusion of radicals from an analogous manganese(IV) porphyrin complex has been proposed by Hill.[30]

We have presented elsewhere evidence for the radical nature of aliphatic hydroxylations catalyzed by iron porphyrin/iodosylarene systems.[31] The results described here reinforce this view and indicate in addition that hydroxylation with partial rearrangement of the allylic group is a characteristic of oxygen transfer by this oxoiron(V) equivalent.[32]

The reactive species in the cytochrome P-450 cycle has not been observed. Accordingly, the nature of any intermediate must be inferred from reactions with substrate molecules. Since the partial allylic rearrangement behavior observed with the enzymic oxidations is similar to that observed with the model iron porphyrin system, the generation of such an oxoiron(IV) porphyrin cation radical is implicated in the enzymic cycle as well. Further, since similar results are observed for the oxygen-dependent, peroxide-dependent, and iodosylarene-dependent P-450 systems, the same active species is probably generated in each case. Minor differences in product distributions would be attributed in such a case to the presence of the alcohol or iodoarene fragment at the active site.[10] Thus, the oxygen rebound mechanism we have described for aliphatic hydroxylation by simple iron peroxide systems[8,12] suffices to explain the cytochrome P-450 cycle as well.

Experimental Section

Electrophoretically homogeneous cytochrome P-450$_{LM2}$, cytochrome P-450 reductase, and hepatic microsomes of phenobarbital-induced rabbits prepared according to published procedures[32] were obtained from the laboratories of M. J. Coon. Concentrations of P-450$_{LM2}$ were determined by measuring the absorbance at 451 nm for the ferrous carbonyl form of the enzyme (ϵ = 110 mM^{-1} cm^{-1}). Typical preparations had specific contents of 15 nmol of P-450$_{LM2}$ per mg of protein. Dilauroylglyceryl-3-phosphorylcholine was purchased from Calbiochem. Cumene hydroperoxide (CHP) and *tert*-butyl hydroperoxide were purchased from Aldrich Chemical Co. and standardized by iodometric titration. Iodosylbenzene was prepared as we have described elsewhere.[12b] 3,3,6,6-Tetradeuteriocyclohexene was purchased from Merck and was determined to be >98%-d_4 by examination of the mass spectrum of the bromine adduct.[10] The position of deuteration was apparent from the complete lack of allylic hydrogens in the 360-MHz proton NMR spectrum. Methylenecyclohexane and β-pinene were purchased from Aldrich Chemical Co., distilled, and filtered through basic alumina (activity I) to remove oxygenated impurities. Proton NMR and VPC analysis of the purified hydrocarbons indicated the complete lack of methylcyclohexene (δ 5.45) and α-pinene (δ 0.9) in these samples.

Peroxide Dependent Hydrocarbon Oxidations with P-450$_{LM2}$. A solution containing 1 nmol of P-450$_{LM2}$, dilauroylglyceryl-3-phosphorylcholine (50 μg), substrate hydrocarbon (5 μL of a 1 M solution in methanol), and potassium phosphate buffer (0.5 mL, 0.2 M, pH 7.4) was made up to 0.95 mL with distilled water. Cumeme hydroperoxide or *tert*-butyl hydroperoxide (50 μL of a 20-mM solution in 1:1 methanol–water) was added, and the reaction mixture was allowed to stand for 15 min at 20 °C. The reaction was quenched by the addition of 100 μL of 30% NaOH saturated with sodium dithionite. The products were extracted with three 1-mL portions of ether. The combined ether extracts were dried over potassium carbonate and reduced in volume to 50–100 μL with a stream of nitrogen. Controls indicated negligible loss of oxidized products when the nitrogen was passed through a J-tipped capillary tube to direct the nitrogen stream *away* from the surface of the ether. Identification and quantitative analysis of the reaction mixtures were performed by GC (15% Carbowax 20 M on Chromosorb W) and GC–MS (10% Carbowax 20 M) analysis of the concentrated ether solutions. In all cases, product analyses were verified by comparisons of the GC–MS behavior of authentic samples.

Hydrocarbon Oxidations with Reconstituted P-450$_{LM2}$ Systems. A solution containing 1 nmol of P-450$_{LM2}$, 2 nmol of cytochrome P-450 reductase, dilaurylglyceryl-3-phosphorylcholine (50 μg), substrate hydrocarbon (5 μL of a 1 M solution in methanol), and potassium phosphate buffer (0.5 mL, 0.2 M, pH 7.4) was made up to 0.85 mL with distilled water and maintained at 20 °C. The oxidation was initiated by the addition of 1.5 mg of NADPH in 150 μL of water. After 15 min, the reaction was quenched by the addition of 100 μL of 30% NaOH. Products were isolated by ether extraction and identified as described above.

Hydrocarbon Oxidation with Hepatic Microsomes. A solution containing PB-induced hepatic microsomes[32] (equivalent of 1 nmol of P-450$_{LM2}$), potassium phosphate buffer (0.5 mL, 0.2 M, pH 7.4), and 5 μL of a 1 M solution of substrate hydrocarbon was maintained at 37 °C for 5 min. Peroxide-dependent oxidations were initiated by the addition of cumene hydroperoxide or *tert*-butyl hydroperoxide (50 μL of a 20-mM solution in 1:1 methanol–water). After 20 min, the reaction was quenched with sodium hydroxide and sodium dithionite and the products were isolated by extraction as described above. Microsomal oxidations with NADPH and oxygen were initiated by the addition of 1.5 mg NADPH in 150 μL of water. After 20 min at 37 °C, the reaction was quenched with 100 μL of 30% NaOH and worked up as before.

Hydrocarbon Oxidation by Metalloporphyrin Model Systems. Chloro(tetraphenylporphyrinato)iron(III) or chloro(tetraphenylporphyrinato)chromium(III) (0.4 mg) and the hydrocarbon substrate (10 μL of 0.1 mM solution in methylene chloride) were dissolved in 150 μL of methylene chloride. The resulting mixture was stirred at room temperature under nitrogen for 15 min. Iodosylbenzene (1 mg, 450 μmol) was added all at once and the mixture was stirred for 45 min. Quantitative analysis of the reaction mixture was achieved by direct GC–MS analysis of the reaction mixture. Products were isolated from the porphyrin catalysts by evaporation of the methylene chloride with a stream of nitrogen and extraction of the residue with ether. Iodosylmesitylene was used in the oxidations of methylenecyclohexane because iodobenzene interfered with the GC assay of the products in this case.

Oxidation of Cyclohexenols with Manganese Dioxide. The ether solutions of cyclohexenols obtained by the enzymic and porphyrin-catalyzed oxidation of cyclohexene-d_4 were stirred with 5–15 mg of activated (black) manganese dioxide at room temperature for 30 min. GC–MS analysis of the reaction mixture indicated complete conversion of the cyclohexenol to 2-cyclohexenone. The deuterium content of the cyclohexenone was determined by analysis of the extremely simple parent and parent minus ethylene regions of the mass spectrum. When this procedure was carried out with 1-deuterio-2-cyclohexen-1-ol, the resulting cyclohexenone contained no deuterium.

Hydrocarbon Oxidation with Selenium Dioxide. Selenium dioxide (4.5 mg, 0.0408 mmol) was added to a mixture of 125 μL of 1-butanol and 25 μL of pyridine. The mixture was stirred at room temperature for 30 min to assure complete dissolution of the selenium dioxide. The hydrocarbon substrate (10 μL) was added and the mixture was stirred at room temperature for 12 h. Analysis of the products was performed as described above. Other procedures[17] involving selenium dioxide produced more rearrangement.

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