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A Biomimetic Study of Cytochrome P450 Related Oxidations of Toluenes Using Synthetic Hemin

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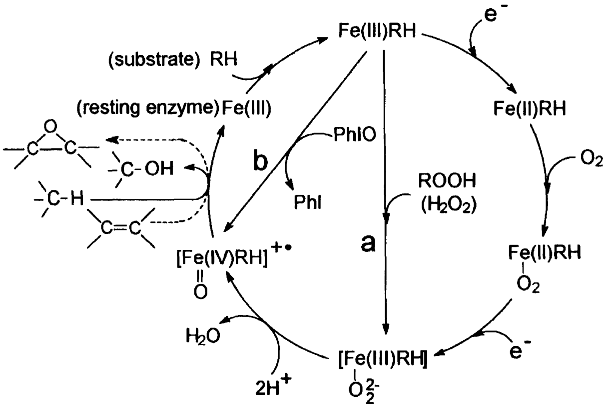
A mechanistic study of the oxidation of toluene and its derivatives in a model system designed to mimic cytochrome P450 (P450) using iron tetrakis(2,6-dichlorophenyl)porphyrin chloride ($FeTPPCl_8Cl$) and pentafluoroiodosylbenzene (PFIB:C_6F_5IO) in dichloromethane is reported. The oxidation products were the corresponding benzyl alcohols and aldehydes; no further oxidation products were observed. The relative reaction rates were obtained in competitive reactions including the use of deuterated analogues. In the present model system, Hammett plots and isotope effects indicated conclusively that the rate-determining step was a hydrogen atom abstraction to the oxoiron intermediate which was then followed by a rebound of an OH group. The concerted mechanism and the rebound mechanism were discussed in terms of the differences in reactivities between the model system and natural P450 enzymes and similar monooxygenation enzymes.

Cytochromes P450 are a group of heme enzymes which have been observed, in microsomes, to catalyze a variety of reactions such as carbon hydroxylation, heteroatom release, heteroatom oxygenation, epoxidation of olefins, oxidative group migration and olefinic suicidal inactivation.[1)] P450s are found in all aerobic organisms including plants and microorganisms. Moreover they play important roles in NO syntheses in the nervous system.[2)] Their reactions are of great importance, because P450s play basic roles in metabolizing xenobiotics, such as drugs and carcinogens. An important feature of these enzymes is that they may be induced by xenobiotics. Such induced enzymes will often oxidize many other substrates. The common active center was confirmed to be a protohemin coordinated by a distal thiolate ion of a cysteine residue.[3)] The reaction catalyzed by P450 in the natural system is described as in Scheme 1.

Synthetic model systems using iron porphyrins have been developed to clarify mechanisms of action or to mimic the enzymatic systems.[4)] The most often studied step of the reaction cycle of P450s (Scheme 2) has focused on the nature and activity of the oxidizing intermediate. The intermediate appears to be common to the various P450s and to other heme proteins such as catalase and it has been postulated to be an oxoiron complex: "iron(IV) porphyrin radical cation."[5)] Hydroxylation reactions are the most fully studied for both the natural[1a)] and model systems.[6)] The oxoiron intermediate can be chemically obtained through "shunt" reactions directly from the hemin using oxidants such as organic hydroperoxide,[7,8)] (route a in Scheme 2) or oxygen atom donors such as *m*-chloroperbenzoic acid[4b)] and iodosylbenzene[9)] (route b in Scheme 2).

$$\mathrm{R + NAD(P)H + H^+ + O_2 \xrightarrow[P450]{} RO + H_2O + NAD(P)^+}$$

Scheme 1.



Scheme 2.

We have previously shown that a model system using $FeTPPCl_8Cl$ as catalyst and PFIB as oxidant mimicked the activities of P450.[10)] This biomimetic system was effective in epoxidation[4b,11)] and in alkane hydroxylation,[12)] and it could reproduce suicide inactivation, which resulted in *N*-alkylation of the porphyrin ring.[13)] On the basis of these results, a mechanism involving electron transfer from olefin to the oxoiron complex accounted for many of the P450 reactions with olefins through a common intermediate.

Recent concerns about the toxicity of toluene and its analogues have renewed interest in the metabolism of these compounds, including induction of P450s and their inhibition.[14—16)]

P450s catalyze a variety of reactions with toluenes as substrates, with the position of hydroxylation being carefully controlled by the enzyme, as shown for example in the oxidation of toluene itself.[15] Backes et al. reported two classes of P450s: one with *m*-xylene gave oxygenation at a methyl group to give the corresponding benzyl alcohol and the other with ethylbenzene gave phenol derivatives by hydroxylation of the phenyl ring.[15c] Toftgard et al. also reported *m*-xylene metabolism in rat lung and liver microsomes, indicating that both side chain hydroxylation and phenyl ring oxidation occurred.[15d] White and McCarthy reported rabbit liver oxidized toluene to benzyl alcohol only.[15e] Though the mechanism of hydroxylation to give phenols has not been elucidated with model reactions, it was proposed for the enzymes themselves that initial epoxidation of the aromatic ring occurred, followed by ring opening and an NIH shift.[16]

Experimental

Materials. 5,10,15,20-Tetrakis(2,6-dichlorophenyl)porphyrin (H_2TPPCl_8) was prepared from 2,6-dichlorobenzaldehyde and pyrrole by the method reported by Lindsey et al.[17] Iron insertion into H_2TPPCl_8 was performed as previously reported.[18] Pentafluoroiodosylbenzene (PFIB)[19] was made by hydrolysis of pentafluoroiodobenzene bis(trifluoroacetate) with saturated sodium hydrogen carbonate. The resulting precipitate was collected and air-dried. Toluenes were purchased commercially and were passed through a SiO_2 column just before the reaction to remove oxidized impurities. Deuterated compounds were obtained from MSD Isotopes: $C_6D_5(CD_3)$ (99.6% D), $C_6H_5CH(CD_3)_2$ (99.5% D) and $C_6H_5CD(CD_3)_2$ (99.3% D). Other chemicals were of reagent grade. Hitachi 220A spectrometer and Shimadzu GC-12A gas chromatograph (PEG 20M and OV-101) were used.

Reactions. (1) In a typical procedure, the reaction was carried out with suspension of oxidant, PFIB. PFIB (2.0 mg: final concentration: $0.065\ \mathrm{mol\,dm^{-3}}$) was charged into a small test tube (4×55 mm) with a silicone stopper, and the corresponding amounts of dichloromethane and neat substrate or substrate solution (final: $2.0\ \mathrm{mol\,dm^{-3}}$) were injected to give a final volume of 100 μl after addition of a catalyst solution (final: $10^{-4}\ \mathrm{mol\,dm^{-3}}$) in CH_2Cl_2. The reaction was started by injecting the catalyst solution into the reaction tube. The reaction was carried out at ambient temperature (25 °C). When the turbidity of the reaction mixture (greenish brown) turned to a clear brown, after 2—3 min, the reaction tube was cooled with ice water to stop further reaction. The products thus formed were measured by GLC (PEG-20M and OV-101). In the case of substrates of low reactivity, the reaction was stopped before degradation (decolorization) of the catalyst by cooling with ice water (15—20 min). In these cases with nitrotoluenes and cyanotoluenes, we found that unreacted PFIB dissolved in CH_2Cl_2, forming adducts with alcohol, which resulted in aldehydes in the injection port of GC. The corrected results were obtained by treating the reacted mixtures with sodium dithionite. Though the present conditions were heterogeneous, the system gave similar results (but a smaller amount of products) to hydroxylation which was carried out in a homogeneous system in the solution of dichloromethane/methanol/H_2O.[20]

(2) The yields obtained from GLC were calculated by comparing to pentafluoroiodobenzene as an internal standard.

(3) Competitive reactions between two substrates were carried out under heterogeneous conditions (total of combined initial concentrations was adjusted to be $2.0\ \mathrm{mol\,dm^{-3}}$). The relative reaction rates were obtained by comparing the products from a particular substrate with the reference: Actually *p*-xylene was used as the reference, except for the cases where retention times of products overlapped on GLC. The data of rates calculated relative to that of toluene oxidation are listed in Table 1.

(4) Isotope effects were obtained using deuterated toluene, $C_6D_5(CD_3)$, and deuterated cumenes, $C_6H_5CH(CD_3)_2$ and $C_6H_5CD(CD_3)_2$, in similar manners competitively as above. *p*-Xylene was used as a reference substrate, and the isotope effects were calculated from the product ratios.

Results and Discussion

Limited Formation of Products. The oxidation of toluene and its derivatives were carried out for substituents over the range of Hammett's σ parameters known. Product yields of the oxidation reactions for toluenes are summarized in Fig. 1. The product yields were based on the PFIB consumed during the reactions. The yields achieved in the present system were higher than those previously reported for model reactions using hemin, FeTPPCl, and iodosylbenzene.[21,22]

The major products were corresponding benzyl alcohols and benzaldehydes or ketones (Scheme 3). The product yields were good for substrates with electron-donating groups, but only modest for those with electron-withdrawing groups. It was shown that, for three isomers of toluene, the product yields were in the order of *p*->*o*- >*m*-isomers for the methyl and halogen derivatives. The amounts of benzaldehydes were about 5—25 % of benzyl alcohols in methyl and halogen derivatives. The coproduction of benzaldehydes was also observed in previous experiments.[22]

Further oxidation products, such as benzoic acid, and benzene ring oxidation products, phenols or cresols, were not observed. Benzene was not detected, excluding the possible formation of benzoic acid and subsequent decarboxylation. No 1,2-diphenylethane was observed during the present oxidations. No demethylation products from anisoles were detected. Influence of air during the present oxidations was not observed.

Though the yields of alcohols and aldehydes were better than those in previous works, the stoichiometry of the

Table 1. Relative Reaction Rates in Competitive Reactions

Substrate	Relative reaction rate[a]
Toluene	1
p-Xylene	3.17
m-Xylene	2.20
p-Fluorotoluene	1.17
m-Fluorotoluene	0.615
p-Chlorotoluene	1.006
m-Chlorotoluene	0.569
p-Bromotoluene	0.613
m-Bromotoluene	0.404
p-Methoxytoluene	8.94
p-Tolunitrile	0.261
m-Tolunitrile	0.282
Mesitylene	3.08

a) Relative reaction rates were k/k_0, where k stands for the rate constant for substrate and k_0 for toluene.

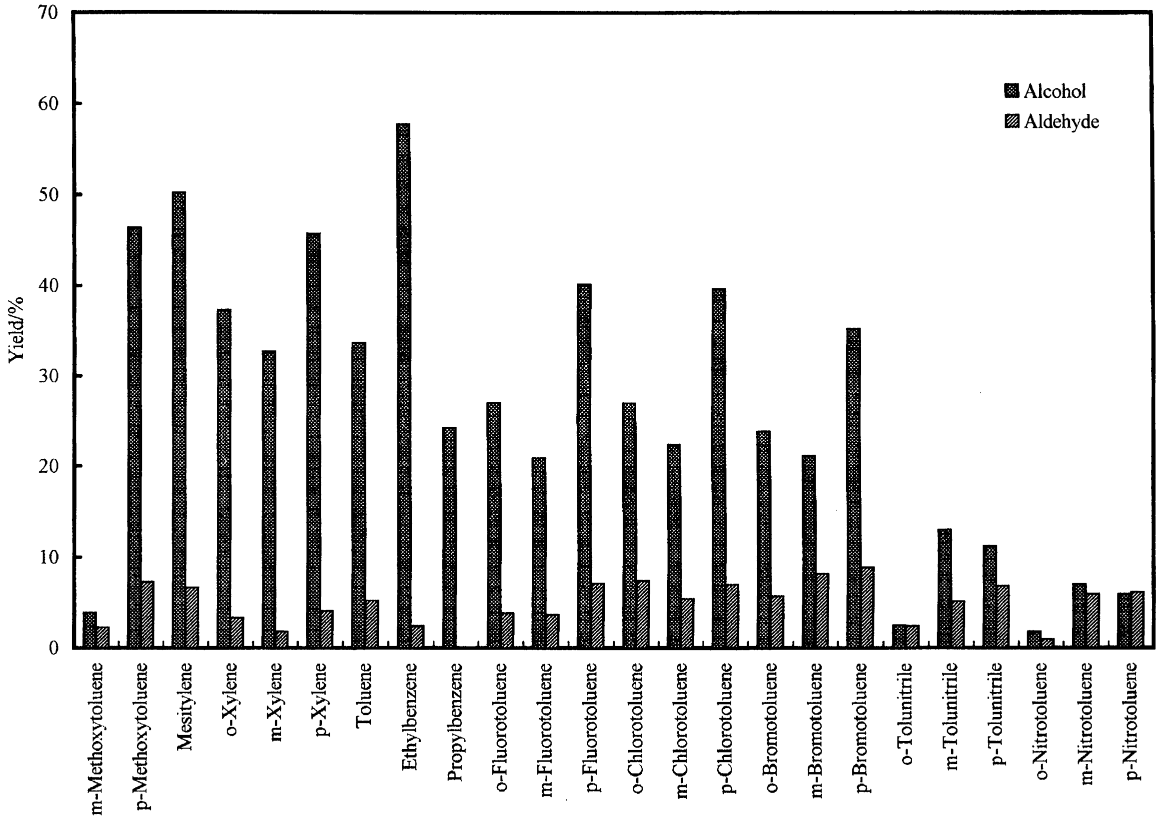
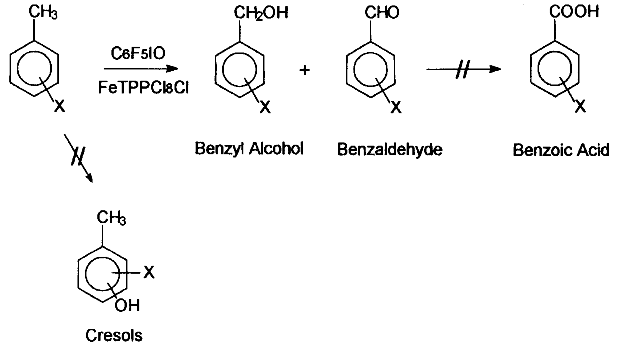


Fig. 1. Product yields in hydroxylation of toluene and its derivatives. Yields (%) were based on the PFIB consumed.



Scheme 3.

reaction in Scheme 3 was not satisfactorily achieved. The oxidant PFIB was not fully consumed during the formation of alcohols and aldehydes, but no other oxidation products were detected. The fate of oxygen from PFIB could not be imagined at this point, but it was used only for a slight damage on the catalyst hemin in the cases of substrates with electron withdrawing groups. Exceptionally, low yield was observed in the *m*-methoxytoluene case and no product was detected in the *o*-methoxytoluene case: No idea to solve the situation was presented at this point.

It was noted that the limited kinds of products were observed here; hydroxylation on side chain of toluenes occurred and no further oxidation occurred but aldehyde was formed. The situation indicated that the reactions reflected only the chemical reactivities: Methyl groups of the toluene derivatives were more reactive than the benzene ring.[23,24)]

We use benzyl alcohol plus benzaldehyde to estimate the relative reactivities of substrates, as the latter is the further oxidation product of the former.[25)]

$$\text{toluene} \xrightarrow{k_1} \text{benzyl alcohol} \xrightarrow{k_2} \text{benzaldehyde} \qquad (1)$$

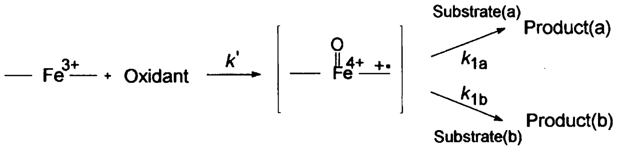
This approximation is valid in those cases where there is little over-oxidation and $k_1 \gg k_2$ or when $k_2 \gg k_1$ and only some benzaldehyde is seen. However, when $k_1 \approx k_2$, this approximation is no longer valid. The fit of the data allows this approximation to be used in the present study. Under the conditions used, the substrates were in a large excess to oxidant and catalyst.

Competitive Oxidations and Electronic Effects of Substituents. As PFIB did not dissolve in CH_2Cl_2, we carried out the reactions under the heterogeneous conditions; the absolute rates needed in order to examine the effect of substituents on substrates could not be determined directly and conveniently. So in the present model system, we chose the relative rate method of competition between two substrates.

The reactions were assumed to occur as in Scheme 4 and Eq. 2.

$$[\text{Product(a)}]_f/[\text{Product(b)}]_f = k_{1a}[\text{Substrate(a)}]_0/k_{1b}[\text{Substrate(b)}]_0 \qquad (2)$$

where subscripts a and b indicate the independent substrates (Scheme 4): f means the final concentration and 0 means the initial concentrations. Products were estimated as the sums of the corresponding benzyl alcohols and benzalde-



Scheme 4.

hydes. The resulting relative reaction rate, k_{1a}/k_{1b}, estimated using Eq. 2 are summarized in Table 1. The data were standardized to be the ratios to toluene, $k/k_0=k_{1a}/k_{1b}$: k_0 was for toluene. Reactivities of nitrotoluenes were too low to obtain meaningful results in competitions.

The data, $\log (k/k_0)$, were plotted against Hammett's σ[26) and σ^+[27) parameters, as shown in Figs. 2 and 3. The linear relationships indicated that the substituents on the benzene ring affected the benzylic hydroxylations of toluenes. The ρ and ρ^+ values were -1.45 and -1.12 respectively, and the correlation factors (r) were 0.954 and 0.977 for σ and σ^+, respectively. The ρ and ρ^+ values observed clearly showed the electronic dependency on substituents: the value for ρ^+ is in a range similar to that of the previous experiment ($\rho^+=-0.83$).22)

Though the relationship between relative rate with σ^+ was slightly better than that with σ in the present study, there was no clear evidence to support a mechanism where an electron transfer from the benzyl radical to generate a benzylium ion in the reaction pathway occurred. Inchley et al.22) reported in a competitive oxidation using Fe(III)TPPCl/iodosylbenzene and five different toluene derivatives in benzene solution that the reaction showed a reasonable linear correlation for σ^+ using a single parameter Hammett equation and a better fit for σ. They tried the dual parameter analyses of $\rho^+\sigma^++\rho\cdot\sigma\cdot$28) using σ^+ and $\sigma\cdot$,29) and suggested that the transition state partially included an electron transfer process as shown in Scheme 5. However, in the present system, we obtained only a slightly improved correlation in the dual parameter analyses; the correlation for $\rho\sigma+\rho\cdot\sigma\cdot$ was 0.968 and $\rho^+\sigma^++\rho\cdot\sigma\cdot$ was 0.982, and the single correlation with $\sigma\cdot$ was really poor at 0.327. No conclusive result for this treatment could be achieved on this matter from Hammett relationships.

Kinetic Isotope Effect of Substrates. A rate-determining step in the natural system was not pointed out clearly and, if hydroxylation is not rate determining, the hydroxylation step will not reflect the intermolecular isotope effect. To obtain the effect on the hydroxylation step in this case, people chose the intraisotope effect using partially deuterated toluenes, $C_6H_5CH_nD_{3-n}$ ($n<3$), which needed complex calculations. On the other hand, in the model system with "open"

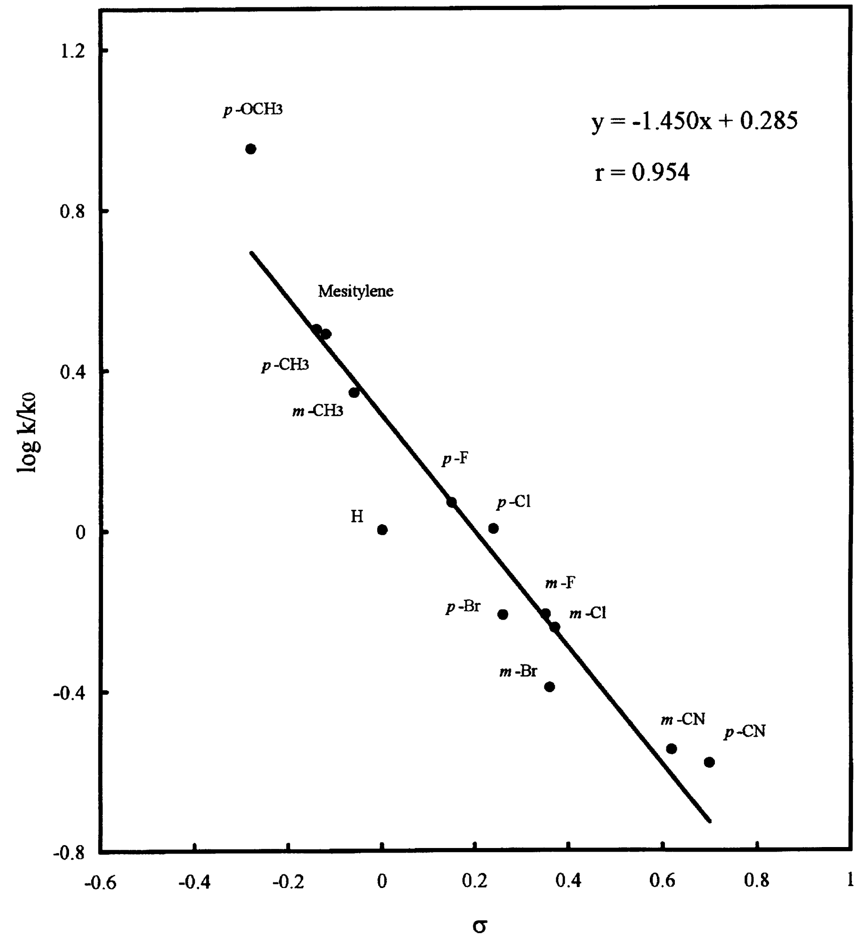


Fig. 2. Hammett plot of hydroxylation of toluene derivatives vs. σ-parameters. The relative rate was estimated against toluene as a standard substrate.

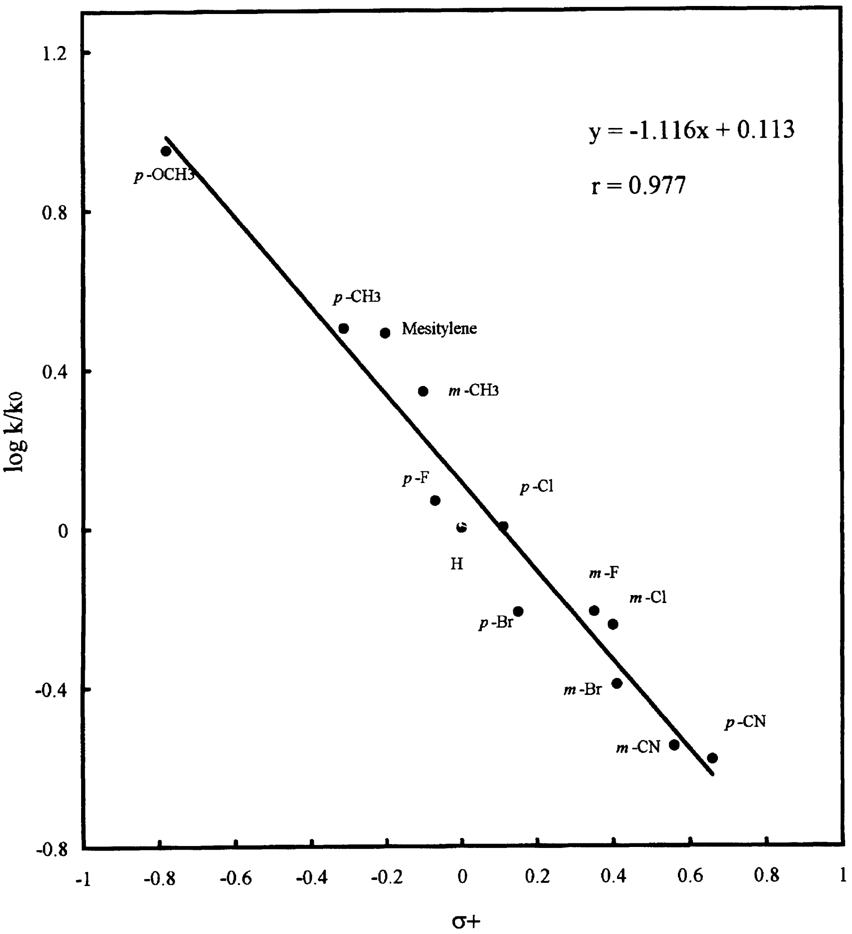
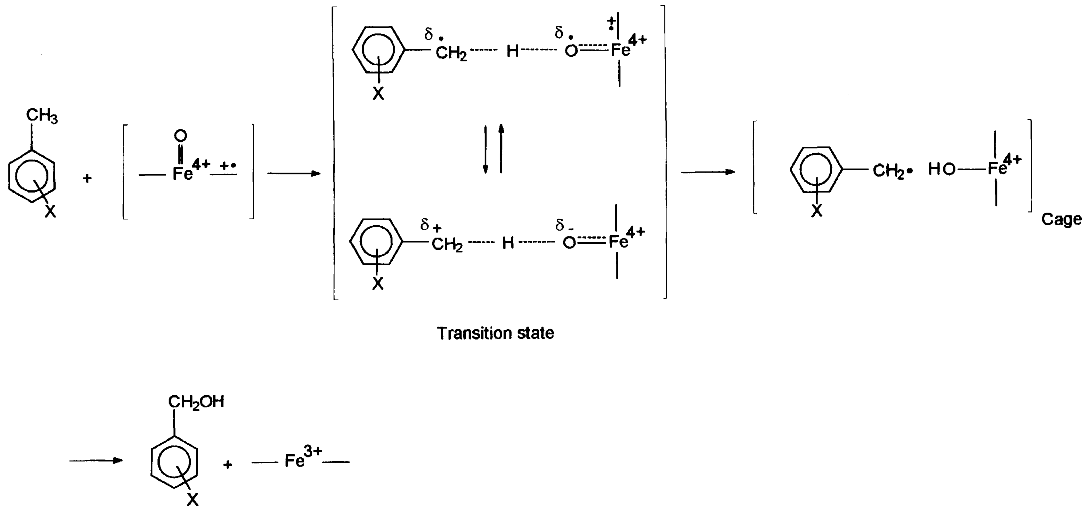


Fig. 3. Hammett plot of hydroxylation of toluene derivatives vs. σ^+-parameters. The relative rate was estimated against toluene as a standard substrate.



Scheme 5.

circumstances, the isotope effect was simply obtained by comparing rates between normal and deuterated substrates. We called a model system "open" when its reactive site was open to reactants without being controlled by a protein or its synthetic analogue.

Yields in competitive reactions between deuterated toluene or partially deuterated cumene and *p*-xylene are summarized in Table 2. *p*-Xylene was chosen to be the actual competing substrate because of its appropriate reactivity and its retention time, allowing for clear separation in the GC measurement. As hydroxylations occurred exclusively on side chains in this toluene system, we obtained an isotope effect from the ratio of the relative rates between $C_6H_5CH_3$ and $C_6D_5(CD_3)$. Similarly, for cumenes, hydroxylation oc-

Table 2. Product Yields in Competitive Reactions between Deuterated Substrates[a] and *p*-Xylene

	Substrate	Corresponding benzyl alcohol[b]	Corresponding benzaldehyde[b]
1)	Toluene	9.39	1.71
	p-Xylene	16.8	2.63
2)	$C_6D_5(CD_3)$	1.76	0.32
	p-Xylene	19.2	3.35
3)	Cumene	7.76	—[c]
	p-Xylene	20.4	3.72
4)	$C_6D_5CH(CD_3)_2$	11.0	—[c]
	p-Xylene	20.0	4.12
5)	$C_6H_5CD(CD_3)_2$	1.94	—[c]
	p-Xylene	20.1	3.58

a) Each row indicated the results using two substrates competitively. b) Yields (%) were based on C_6F_5IO consumed. c) No ketones formed.

curred only at a benzylic position to give cumene hydroxide, a situation which allowed us to determine the isotope effect directly using partially deuterated cumenes. The data are listed in Table 3 along with those in the literature.

The deuterium isotope effects were 6.21 for toluene obtained between $C_6H_5CH_3$ and $C_6D_5(CD_3)$ and these included both primary and α secondary effect which could not be separated here. The primary isotope effect of benzylic hydroxylation for cumene was formed to be 5.71 for the ratio of $k_{C_6H_5C\underline{H}(CD_3)_2}/k_{C_6H_5C\underline{D}(CD_3)_2}$. The *β* isotope effect in this system was 0.71 for the ratio of $k_{C_6H_5C\underline{H}(CH_3)_2}/k_{C_6H_5C\underline{H}(CD_3)_2}$. The data obtained here showed clear deuterium isotope effects in the "open" model system.

In general, isotope effects reported in the natural enzyme systems were low (<2.0),[30] with some exceptions. A reported kinetic deuterium isotope effect in toluene oxidation using P450IIB1[31] was 7.4, but some others indicated low numbers for P450LM$_2$,[15e] fungi[32] and chloroperoxiase[33] (Table 3). Values of isotope effects obtained in the model system of chlorination of toluene where hydrogen abstraction was the rate-determining step were 5.90 (primary) and 1.03 (secondary).[34] It should be noted that isotope effects in the model system differ from those in actual enzyme systems.

Table 3. Kinetic Isotope Effects in Hydroxylations of Toluenes, k_H/k_D, and Related Reactions in Literatures

System/ Substrate	Isotope effect: k_H/k_D		References
FeTPPCl$_8$Cl+PFIB/ Toluene	Primary	6.21	This work
FeTPPCl$_8$Cl+PFIB/ Cumene	Primary	5.71	This work
	Secondary	0.71	
P450(P450IIB1)/ Toluene		7.4	31
P450LM$_2$/ Toluene		2.6	15e
Fungi/Toluene	Primary	1.27	32
	Secondary	1.04	
Chloroperoxidase/ *p*-Methylanisole		3.3—3.4	33
Chlorination Model/ Toluene	Primary	5.90	34
	Secondary	1.03	

Mechanisms of Hydroxylation in "Open" Model Systems. What is the mechanism of toluene hydroxylation and what is the rate-determining step? The hydroxylation mechanism was believed to be a concerted process until Groves et al. suggested a stepwise process with a hydrogen atom abstraction, followed by a rebound of the OH group.[1b]

In some model systems such as phenoxy radical formation[35] and ABTS (2,2′-azino-bis(3-ethyl-2,3-dihydrobenzothiazole-6-sulfonic acid)) radical formation[8] the rate-determining step was found to be the formation of the oxo-iron intermediate. Traylor et al. reported that in a homogeneous system the "overall" rate-determining step was the step forming the active intermediate and the reaction rate was independent of substrate structure and concentration.[36] On the other hand, the dependency on substrates were also reported in epoxidation of olefins[36] or in hydroxylation of hydrocarbons.[6c]

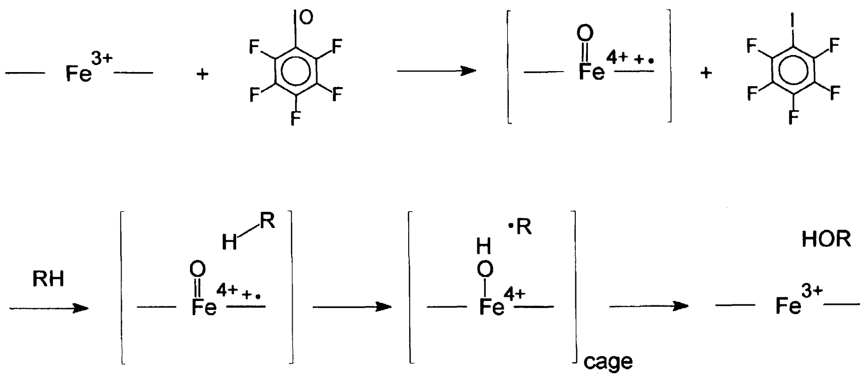
Here, it seemed very clear that the definite deuterium isotope effects and the obvious correlation between relative rates and Hammett parameters indicated that, in the "open" model systems, hydrogen migration played an important role and was affected by electronic properties of substituents on substrates. Thus, so far, the most plausible mechanism for aliphatic hydroxylation is that proposed by Groves as shown in Scheme 6 where a hydrogen atom is initially abstracted and a recombination of hydroxyl radical and substrate radicals, in a cage, occurs.[28,37]

The deuterium isotope effects suggested that the transition state of hydrogen abstraction was in the linear configuration of O–H–C, in a transition state, as shown in Scheme 7a. The reverse *β* secondary deuterium effect is opposed to the existence of carbocation characters in transition states.[38]

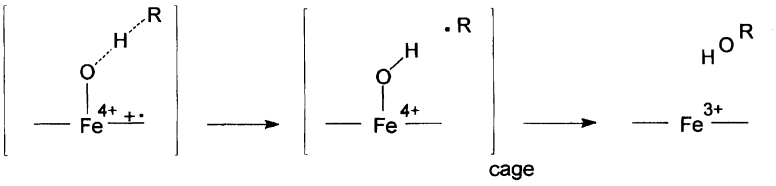
The discrepancy between the model system and the actual enzyme system suggested that the hydroxylation processes were not the same in the natural P450s and the present model system. It could not be said either the hydroxylation step was rate-determining or not, though an intraisotope effect could be observed.

Recently, Newcomb et al. suggested the possibilities of concerted mechanisms for the transition state based on the radical clock measurements in P450CYP2B1 or microsomes using (*trans*,*trans*-2-*t*-butoxy-3-phenylcyclopropyl)methane as a substrate.[39] They pointed out the confused results of clock measurements in P450 enzymes.[40] They argued that the radical clock in hydroxylation was too short, 70 fs, to allow the rebound of OH group to benzylic carbon which was formed as the result of hydrogen abstraction and concluded that concerted and non-synchronous mechanism could explain the phenomena (Scheme 7b).[41]

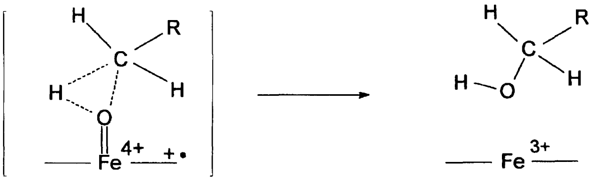
With the actual enzymes in which system isotope effects are small (1—2), there might exist concerted mechanisms. The concerted mechanism cannot be excluded in the actual enzyme system. If the concerted mechanism is operative, it



Scheme 6.



(a) Hydrogen Atom Abstraction and Rebound Mechanism



(b) Concerted Mechanism

Scheme 7.

might be controlled by protein structures and configurations.

Functions in P450s Differing from the Model System. Several reports on the oxidation of toluene derivatives by cytochromes P450 have been made.[42] Artificial model systems could have realized many functions of P450s without being controlled by specific structures of proteins and even without the coordination of thiolate, which discriminated, in actual enzymes, cytochrome P450 from other heme proteins. However, some ambiguity remains in discussing the mechanism in the actual enzyme systems. The complex situation comes from the nature of P450s themselves, since the enzymes are often induced by several different substrates and do not have strict specificity to substrates. In case of toluene oxidations, product distribution is often more complex, with benzoic acid and cresols being formed in addition to benzyl alcohols and benzaldehydes. Moreover, while dinitrotoluenes showed no reactivity in our model system, they are metabolized by hepatic cytochrome P450.[43]

The fundamental idea here is that the chemical data about the mechanism were obtained in the model systems and the modification for the actual enzyme system should be done on the chemical data to treat the mechanism in P450s in different situations. Above discrepancies should be dissolved in these phenomena, relating to the identification of substrates by proteins.

It seemed that the hydroxylation mechanism differs in the model with open system and in the actual enzyme system with an active site. Protein plays a critical role in both orienting the substrate and modulating the redox potential at the active site, with the result that the rate-determining step during the oxidation of toluenes in the natural systems is different from that found in the present studies. These facts indicated that in the "open" model system the rebound mechanisms were favored and in the enzyme systems, concerted mechanisms were favored, depending on how substrates are located in the enzyme pockets. In this context, the proposed concerted mechanism might exist in the actual enzyme systems. Experiments with radical clock measurement would be expected to clarify the situation.

Conclusion

The present study showed that toluene and its derivatives are good substrates for studying the hydroxylation catalyzed by artificial ironporphyrin complexes. The results obtained here indicated that hydroxylation occurred at the side chains (α-position) of toluene derivatives and the reaction rates were dependent on the electronic (donating or withdrawing) effects of substrate substituents, leading the conclusion that the hydroxylation was initiated by abstraction of hydrogen atom and followed by the rebound of OH group in a cage. As in natural P450 systems, the reactivities of toluene and xylene are controlled to give an oxygenated product on both side chains or on phenyl rings as determined by the nature of the active site of the proteins in specific P450s. The difference of mechanisms between artificial models and actual enzymes was important to understand the role of protein which controlled the locations of hydroxylations and rate-determining steps.

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