Mechanisms of Enzymatic *S*-Oxygenation of Thioanisole Derivatives and *O*-Demethylation of Anisole Derivatives Promoted by Both Microsomes and a Reconstituted System with Purified Cytochrome P-450

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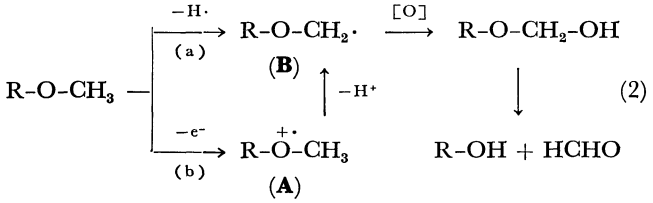
(Received April 13, 1981)

Oxygenations of thioanisole derivatives have been shown to be promoted with phenobarbital induced rabbit liver microsomes. In order to understand the mechanistic details of the oxygenations, a kinetic study on the *S*-oxygenation promoted by a reconstituted system with purified cytochrome P-450 has been carried out. Log(V_{max}) were correlated with the one electron oxidation potentials of the sulfides (E_p). The oxygenation is considered to proceed *via* one electron transfer from the sulfides to the iron-bound "oxenoid" intermediate of the enzyme. A similar *O*-demethylation of anisoles has been investigated. In the oxidative *O*-demethylation of *p*-methoxy-d_3-anisole with rabbit liver microsomes, an intramolecular kinetic isotope effect of 3.4 was observed. Meanwhile, a large isotope effect of 5.1 was observed in the competitive *O*-demethylation of *p*-dimethoxybenzene and *p*-di(methoxy-d_3)benzene. In order to clarify the oxidation pathway of *O*-demethylation with the enzymatic system of cytochrome P-450, the rates of the *O*-demethylation of *p*-substituted anisoles have been measured. Unlike the enzymatic oxygenation of sulfides, there was no correlation between the rates of *O*-demethylation reaction and one electron oxidation potentials of anisoles. These observations suggest that the *O*-demethylation of anisoles proceeds *via* the initial hydrogen abstraction with the iron-bound "oxenoid" intermediate of the cytochrome P-450.

Oxidative *O*-demethylation of methyl ethers catalyzed by the hepatic microsomal cytochrome P-450 system, requiring NADPH and O_2, is one of typical oxidation reactions by mono-oxygenases,[1,2] and numerous substrates have been considered to be demethylated *via* forming the corresponding hemiacetal intermediates (Eq. 1).[3] This reaction involves the

$$\text{R-O-CH}_3 \xrightarrow{\text{enzyme}} [\text{R-O-CH}_2\text{-OH}] \xrightarrow{\text{non-enzyme}} \text{R-OH} + \text{HCHO} \quad (1)$$

initial removal of α-hydrogen and subsequent recombination of the resulting intermediate ((B) in Eq. 2) with the iron-bound "oxenoid" intermediate ($(FeOH)^{III}$) before collapsing to afford the hemiacetal intermediate. Since all the subsequent steps which involve highly reactive intermediates after the initial hydrogen removal would be quite fast, the initial hydrogen removal is presumed to be the rate-determining step of the overall reaction of demethylation. However, the removal of α-hydrogen could proceed *via* a different mechanistic route, as shown below.



If the direct hydrogen-removal (a) is the actual path, one would find a relatively large kinetic isotope effect of α-hydrogen, whereas a small secondary kinetic isotope effect, k_H/k_D, would be observed when the proton removal takes place after the rate-determining electron transfer to form the cation radical (**A**). Thus, the kinetic isotope effect was studied to diagnose the mechanistic pathway of enzymatic demethylations and oxygenations.

In the *O*-demethylation of *o*-nitroanisole,[4] *p*-methoxy-acetanilide,[5] and *p*-dimethoxybenzene[5] and their methyl-d_3 derivatives, mediated by rat liver microsomes, the kinetic isotope effects, *i.e.* k_H/k_D of *ca.* 2 have been observed, whereas in the mono-*O*-demethylation of *p*-methoxy-d_3-anisole a large primary isotope effect ($k_H/k_D=10$)[5] was observed by the intramolecularly competitive reaction. In the *O*-dealkylation o propyl *p*-nitrophenyl ether, however, a rather small kinetic isotope effect ($k_H/k_D=1.1$) was observed during the metabolism with the rat and guinea pig hepatic enzyme systems.[6]

Recently, Hamilton and others postulated an iron-bound "oxenoid" intermediate as the active component species of mono-oxygenases.[7–13] This iron-bound "oxenoid" intermediate has been postulated to be analogous to the Compound I of peroxidase[10,13] and this postulate has been supported by extensive investigation with a few enzyme-model systems.[14,15] In other enzymatic oxidations of hydrocarbons, several *in vitro* measurements of the primary isotope effect give rather low values, such as $k_H/k_D \approx 2.0$.[16–20] In these experiments, kinetic isotope effects, k_H/k_D, were obtained by comparison of the reactivities of the deuterated and the undeuterated substrates, in separate kinetic runs. However, the enzymatic oxidation reaction consists of several succesive steps and hence the measured isotope effects are the summation of some steps of the overall enzymatic oxidation reaction. In order to avoid the complication of the kinetic treatment due to the different pre-oxygenation processes such as an electron transfer reduction of the cytochrome P-450-substrate complex and to estimate the real kinetic isotope effect of the enzymatic hydroxylation of hydrocarbons, it is desirable to measure the kinetic isotope effect of an intramolecularly competitive reaction.

Both Hjelmeland *et al.*[21] and Groves *et al.*[22] ob-

tained large values of $k_H/k_D \doteqdot 11$ for intramolecular isotope effects, using compounds which have both deuterated and undeuterated methylene groups in one molecule in the oxygenation of benzylic carbon and aliphatic carbon. These results suggest that the rate-determining step of the oxygenation is the hydrogen abstraction from hydrocarbons with the iron-bound "oxenoid" intermediate.[22]

Recently we have reported a few typical examples of enzymatic oxygenation of divalent sulfur compounds to the corresponding monoxides by both liver microsomes and its purified cytochrome P-450 system[23,24] and postulated that the oxygenation of sulfides by cytochrome P-450 involves the formation of sulfenium radical as a key intermediate.[25] Since the iron-bound "oxenoid" intermediate is considered to be a higher valent iron-oxo species and hence it would be highly electron deficient, the *S*-oxygenation of sulfur compounds would be initiated by an electron transfer from a divalent sulfide to the iron-bound "oxenoid" intermediate.[26] Meanwhile, we have also shown in the oxygenation of sulfides with a Fenton system[27] that *S*-oxygenation reaction involves generation of a cation radical of the sulfide. These observations on the oxygenation of sulfides suggest that even in the *O*-demethylation of anisole derivatives, the two mechanistic pathways shown in Eq. 2 are conceivable; i) the path (a), direct hydrogen abstraction and subsequent hydroxylation of methyl group (mechanism a), ii) the path (b), one electron transfer process prior to hydroxylation similar to the concurrent *S*-oxygenation and *S*-dealkylation.[25]

This paper gives kinetic evidence to support the one electron transfer mechanism for the *S*-oxygenation of *p*-substituted thioanisoles and to rule out the one electron transfer mechanism for the *O*-demethylation of *p*-substituted anisoles.

Results

Kinetic Experiment on The S-*Oxygenation of* p-*Substituted Thioanisole Derivatives.* When a 40—600 μM of methanolic solution of thioanisole (**1c**) was aerobically incubated in the reconstituted system, containing both purified cytochrome P-450 and NADPH-cytochrome P-450 reductase, oxidation of NADPH was observed. The addition of thioanisole (**1c**) induced a sudden increase of the rate of the NADPH consumption. Similar results were obtained in the oxidation of all the other sulfides (**1a**—**e**). Since a small amount of NADPH consumption was found to be devoured by the substrates even with a complete reconstituted system minus cytochrome P-450, the rates of the oxygenation were calculated by subtracting the rates of these undesired side reactions from the overall rates of NADPH consumption.

The V_{max} and K_m parameters of the oxygenation of sulfides by the reconstituted cytochrome P-450 system calculated from the Lineweaver-Burk plots are listed in Table 1, which also lists the effects of *p*-substituents in the oxygenation of thioanisole derivatives. Except *p*-chlorothioanisole (**1d**), the rate of oxygenation of the sulfide was found to decrease by the electron-withdrawing substituent while V_{max} value of the oxygenation increased by the electron-donating substituent.

Table 1. Kinetics of oxidation of NADPH in the reconstituted system with purified cytochrome P-450

Substrate	K_m (μM)	V_{max}[a] (μM/min)	V_{max}[a] (nmol/min/nmol P-450)	Binding type
1a	63	20	18	I
b	77	19	17	I
c	110	15	14	I
d	135	23	25	I
e	31	12	11	—[b]
3a	39	5.8	5.3	I
b	130	5.7	5.1	I
c	40	6.0	5.4	I
d	75	6.8	6.2	I
e	54	6.5	5.9	—[b]

a) The oxidation of substrate was monitored by the consumption of NADPH. b) **1e** and **3e** have so large absorbances in the region (350—500 nm) that the difference spectra cannot be observed.

Table 2. Comparison of one electron oxidation potential with both Hammett σ^+ and σ

Substrate	E_p *vs.* SCE[a] (V)	Substrate	E_p	σ^+	σ
1a	1.26	**3a**	1.47	−0.78	−0.27
b	1.41	**b**	1.76	−0.17	−0.17
c	1.53	**c**	1.83	0	0
d	1.55	**d**	2.17	0.23	0.23
e	1.85	**e**	2.36	0.79	0.71

a) Oxidation potentials were measured in 0.1 M of n-Bu_4NClO_4/CH_3CN.

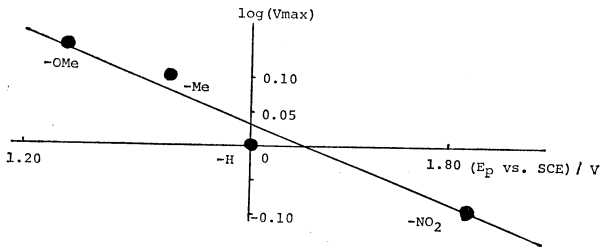


Fig. 1. Relationship between log (V_{max}) and E_p of *p*-substituted phenyl methyl sulfides.

In order to examine the possible relationship between the V_{max}'s of the oxygenation and one electron oxidation potentials of sulfides (**1a**—**e**), the cyclic voltammograms of the sulfides were obtained in acetonitrile (Table 2).

The V_{max} values in Table 1 were found to be correlated better with Hammett σ^+-values rather than σ-values ($\rho^+ = -0.16$), and the one electron oxidation potentials of the sulfides (except **1d**) are also correlated linearly with log(V_{max})'s of the enzymatic oxygenation of the sulfides (Figs. 1 and 2). E_p's (one electron oxidation potential) of the sulfides are con-

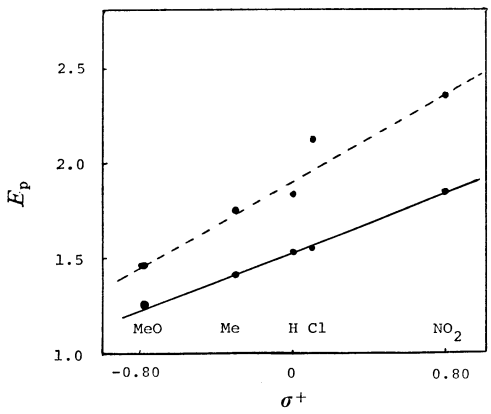


Fig. 2. Correlation between one electron oxidation potentials and Hammett σ^+-values.
----: Ether (**3**), ——sulfide (**1**).

TABLE 3. MICROSOMAL OXIDATION OF SULFIDE (**1**) AND ETHER (**2**)

Substrate	Product	(μmol)[c]	(nmol/min/nmol P-450)
1a[a]	**2a**	9.1	1.4
b	**b**	9.0	1.4
c	**c**	8.2	1.3
d	**d**	11.6	1.8
e	**e**	3.3	0.5
3a[b]	HCHO	34.0	5.3
b		17.7	2.8
c		22.5	3.5
d		21.3	3.3
e		24.4	3.8

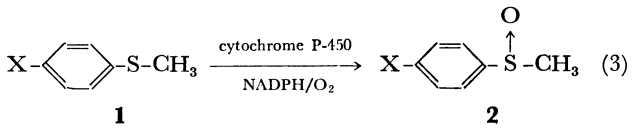
Microsomal oxidation of sulfides and ethers were carried out for 20 min. a) $100 \times K_m$ (see Table 1) of sulfides were incubated in the oxidation. b) 250 μmol of ethers were incubated (see Experimental). c) In the oxidation of sulfides the corresponding sulfoxides were analyzed by GLC, and in the case of ethers yields of formaldehyde were determined by using Nash reagent.

sidered to correspond to the first oxidation peak potentials instead of $E_{1/2}$, due to the irreversible nature of the cyclic voltammograms.[28]

Incidentally, all the sulfides were found to be typical compounds of type I[29] having a maximum at 385—390 nm and a minimum at about 420 nm from the substrate-induced difference spectrum.

A methanolic solution of thioanisole (**1c**, 260 μmol, $100 \times K_m$) was added into a phosphate buffer (0.2 M, pH 7.4) containing both NADPH generating system and rabbit liver microsomes (see Experimental) and the incubation was carried out at 37 °C for 20 min with stirring. The oxygenation was stopped by adding 20 ml of acetone and 5 ml of trichloroacetic acid (0.5 M)† into the solution. After centrifugation of the reaction mixture, the supernatant solution was extracted with chloroform and the chloroform solution was dried over anhydrous magnesium sulfate. The product analysis was performed by GLC, and the sulfoxide (**2c**) was obtained as the sole product in

† 1 M = 1 mol dm^{-3}.



1 **2** (3)

X: a, MeO b, Me c, H d, Cl e, NO_2

the microsomal oxygenation. Similar results were obtained with all the sulfides used (**1a—e**) (Table 3).

Kinetic Experiment on The O-Demethylation of p-*Substituted Anisole Derivatives.* Rates of the *O*-demethylation of *p*-substituted anisoles (**3a—e**) were measured according to the method described in the *S*-oxygenation. The K_m and V_{max} values of oxygenation of anisole derivatives (**3a—e**) in Table 1 show clearly that all the substrates have similar V_{max}'s values. Unlike the *S*-oxygenation or *S*-dealkylation, $\log(V_{max})$'s of enzymatic oxidations of the ethers cannot be correlated with either Hammett σ-values, σ^+-values or with one electron oxidation potentials (Fig. 3).

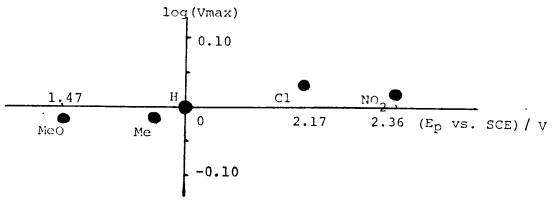
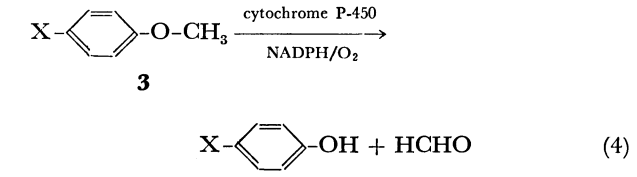


Fig. 3. Relationship between $\log(V_{max})$ and oxidation potential of *p*-substituted anisols.

On the other hand, one electron oxidation potentials of ethers (**3a—e**) in acetonitrile were correlated linearly with Hammett σ^+-values (Table 2 and Fig. 2).

The oxygenation of ethers (**3a—e**) by the reconstituted system of purified cytochrome P-450 gave *p*-substituted phenols (**4a—e**) and formadehyde. Oxygenation of anisole derivatives (**3a—e**) by the micro-



3 (4)

X: a, MeO b, Me c, H d, Cl e, NO_2

somal system were also performed similarly. After aerobical incubation, microsomal protein was removed from the mixture and formaldehyde formed was assayed using the Nash reagent,[30] and the extract was acidified with 2 M HCl and extracted with chloroform to obtain the other products such as phenols. The residure was analyzed by GLC and TLC. Only *p*-substituted phenols were obtained as the sole oxygenation product. In Table 3, the amounts of formaldehyde generated in the enzymatic reactions are listed.

O-Demethylation of p-*Substituted Anisoles and Its Methyl-d_3 Analogues.* When *p*-substituted anisole (**3**, 250 μmol) was aerobically incubated with the microsomes for 20 min, *O*-demethylation takes place as described in Table 3. Similarly, its methyl-d_3 analogue (**3**-d_3) was also oxygenated by the microsomes to give for-

Table 4. Intermolecular isotope effect by deuterium substitution on the metabolism of anisole by rabbit liver microsomes

Substrate		HCHO (μmol)	k_H/k_D[a]
Cl-C$_6$H$_4$-O-CH$_3$	**3d**	21.3	
Cl-C$_6$H$_4$-O-CD$_3$	**3d**-d_3	19.5	1.1
CH$_3$O-C$_6$H$_4$-OCH$_3$	**3a**	34.0	
CH$_3$O-C$_6$H$_4$-OCD$_3$	**3a**-d_3	32.1	1.1
CD$_3$O-C$_6$H$_4$-OCD$_3$	**3a**-d_6	15.9	2.1

a) $k_H/k_D = [HCHO]_H/[HCHO]_D$.

maldehyde and *p*-substituted phenol (**4**). (Table 4) This kinetic isotope effect, between deuterated and undeuterated anisoles, was 1.1 by the comparison of the amounts of formaldehyde. In the oxygenation of *p*-di(methoxy-d_3)benzene (**3a**-d_6), the kinetic isotope effect of 2.1 was observed.

When a solution of *p*-methoxy-d_3-anisole (**3a**-d_3) was incubated with the microsomes for three different periods (20, 60, and 90 min), the ^{1}H-FT-NMR spectrum of the mixture of **4a** and **4a**-d_3 was measured at 20—40 pulses. Since the chemical shifts of aromatic ring protons of **4a** and **4a**-d_3 appear at 6.78 ppm (δ-value, 4H,s) while that of methoxyl protons of **4a** is found at 3.76 ppm (3H, s) in $CDCl_3$, the ratio of the two products **4a** and **4a**-d_3 at three periods is given by Eq. 5.

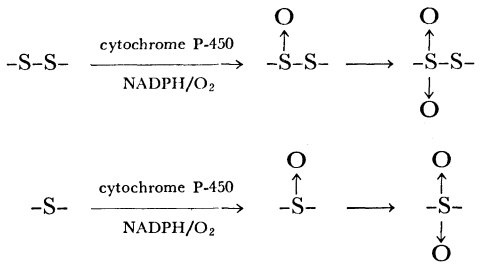
$$[\mathbf{4a}]/[\mathbf{4a}\text{-}d_3] = \frac{[\text{methoxyl proton}]/3}{[(\text{aromatic ring proton}) - 4/3(\text{methoxyl proton})]/4} \quad (5)$$

From the intensities of the two singlet peaks of ethers, the ratios (*e.g.* **4a**:**4a**-d_3=23:77) were obtained (Table 5). From the ratio between **4a** and **4a**-d_3, the genuine kinetic isotope effect (k_H/k_D=3.4) for the *O*-demethylation was estimated.

A mixture of *p*-dimethoxybenzene (**3a**) and *p*-di-(methoxy-d_3)benzene (**3a**-d_6) (1:1) was incubated with the microsomes for 20 min. In the intermolecularly competitive reaction, a kinetic isotope effect of 5.1 was obtained from ^{1}H-FT-NMR. (Table 6).

Discussion

We have recently reported a few examples of the enzymatic oxygenation of divalent sulfur compounds to the corresponding monooxides by both rabbit liver microsomes and a reconstituted system with the purified cytochrome P-450.[23,24] We have also shown



(6)

that sulfides are good substrates for the oxygenation with cytochrome P-450 enzyme and are readily oxidized to the corresponding sulfoxides and even further to sulfones,[25] or undergo *S*-dealkylation, indicating clearly that there are two reaction pathways in the enzymatic oxygenation of sulfides;[26] one is the *S*-dealkylation of sulfides to give thiols (or further oxidized disulfides) and aldehydes while the other is the ordinary *S*-oxygenation, as illustrated in Eq. 7. These two types of reactions appear to take place simultaneously while the acidity of α-hydrogen of alkyl

Table 5. Intramolecular isotope effect of deuterium substitution of *p*-dimethoxybenzene by the rabbit liver microsomes

CH$_3$O-C$_6$H$_4$-OCD$_3$ **3a**-d_3	CH$_3$O-C$_6$H$_4$-OH **4a**	CD$_3$O-C$_6$H$_4$-OH **4a**-d_3	k_H/k_D
Incubation time/min	Product ratio[a]		
20	23 : 77		3.4
60	23 : 77		3.4
90	24 : 76		3.2

a) Product ratio was measured by ^{1}H-FT-NMR spectrum.

Table 6. Competitive *O*-demethylation of *p*-dimethoxybenzene (**3a**-d_6 and **3a**) by rabbit liver microsomes

Substrates	Products	CD$_3$O-C$_6$H$_4$-OH **4a**-d_3	CH$_3$O-C$_6$H$_4$-OH **4a**	k_H/k_D
CD$_3$O-C$_6$H$_4$-OCD$_3$ **3a**-d_6[a]				
CH$_3$O-C$_6$H$_4$-OCH$_3$ **3a**[a]		84 :	16[b]	5.1

a) The mixture of **3a** (108 μmol) and **3a**-d_6 (108 μmol) was incubated for 20 min. b) Product ratio was determined by ^{1}H-FT-NMR spectrum.

sulfides and the stability of α-sulfenyl radicals seem to play important roles in determining the mode of the enzymatic oxygenation of sulfides, similar to the

$$\mathrm{R\text{-}S\text{-}CH_2R'} \xrightarrow[\mathrm{NADPH/O_2}]{\text{cytchrome P-450}} \begin{cases} \rightarrow \mathrm{R\text{-}\overset{\uparrow}{\underset{}{S}}(O)\text{-}CH_2R'} \\ \quad (S\text{-oxygenation}) \\ \rightarrow 1/2\ \mathrm{R\text{-}S\text{-}S\text{-}R} + \mathrm{OHCR'} \\ \quad (S\text{-dealkylation}) \end{cases} \quad (7)$$

R=aryl, R'=alkyl.

N-oxygenation and *N*-dealkylation of tertiary amines with enzymatic and biomimetic model systems.[31,32] In the reaction of alkyl sulfides with hydroxyl radical generated by the Fenton's reagent,[27] a similar mode of oxidation reaction was observed. The initial step of this reaction is known to be the generation of sulfenium radical intermediate[33] (Eq. 8). Thus the similar mode of reaction and of products distribution suggests strongly that the enzymatic oxygenation of sulfide with cytochrome P-450 involves the formation of the cation radical intermediate.[25]

In order to confirm that the enzymatic oxygenation also proceeds through one electron transfer process, a kinetic study on the *S*-oxygenation promoted by the reconstituted system with purified cytochrome P-450 was performed by following the disappearence of the UV absorption of NADPH, since we had already shown that the rates of oxidation of NADPH in the

$$\mathrm{R\text{-}S\text{-}CH_2R'} + \cdot\mathrm{OH} \rightarrow \underset{\text{sulfenium radical}}{[\mathrm{R\text{-}\overset{+\cdot}{S}\text{-}CH_2R'}]} \begin{cases} \xrightarrow{\cdot OH} \mathrm{R\text{-}\overset{\uparrow}{\underset{}{S}}(O)\text{-}CH_2R'} \\ \xrightarrow{-H^+} \mathrm{R\text{-}S\text{-}\dot{C}HR'} \end{cases} \quad (8)$$

$$\xrightarrow{\cdot OH} \mathrm{R\text{-}S\text{-}\underset{OH}{\underset{|}{C}}HR'} \longrightarrow \mathrm{R\text{-}SH}\ (+\mathrm{OHCR'})$$

$$\longrightarrow 1/2\ \mathrm{R\text{-}S\text{-}S\text{-}R}$$

R=aryl, R'=Ph, CN, COPh *etc.*

reconstituted system with cytochrome P-450 are proportional to that of the generation of the sulfoxide.[23] Fine correlations between $\log(V_{max})$'s of the oxygenation of sulfides (**1a**—**e**) except (**1d**)[34] and both one electron oxidation potentials and Hammett σ^+-values are illustrated in Table 1 and Fig. 1. While the rates of the oxidation of these sulfides with hydrogen peroxide are correlated nicely with Hammett σ-values ($\rho=-1.17$),[35] V_{max}'s in Table 1 are correlated better with σ^+-values than σ-values ($\rho^+=-0.16$). These data cannot be explained on the basis of the simple nucleophilic attack of divalent sulfur atom of the sulfide on the electrophilic center of the iron-bound "oxenoid" intermediate, unlike the oxidation of sulfides with hydrogen peroxide. The good correlation between one electron oxidation potentials and the oxygenation rates of sulfides mediated by cytochrome P-450 system suggests clearly that the *S*-oxygenation is initiated by one electron transfer from the divalent sulfide to the iron-bound "oxenoid" intermediate of cytochrome P-450, as shown below.

$$\mathrm{Ar\text{-}S\text{-}CH_3} \xrightarrow{\text{cytochrome P-450 } (FeO)^{3+}} \mathrm{Ar\text{-}\overset{+\cdot}{S}\text{-}CH_3} + (FeO)^{2+}$$

$$\longrightarrow \mathrm{Ar\text{-}\overset{\uparrow}{\underset{}{S}}(O)\text{-}CH_3} + \text{cyt. P-450 } Fe^{3+} \quad (9)$$

The relatively small substituent effect on the V_{max} can be rationalized in term of the extremely facile nature of the one electron transfer process because of the highly electron-demanding character of the iron-bound "oxenoid" intermediate.[36]

In the enzymatic oxidative *N*-demethylation of tertiary amines, similar cation radical intermediates have been considered to be involved and the oxidation of aminopyrine by horseradish peroxidase has also been suggested to involve the cation radical intermediate on the basis of the ESR spectroscopic studies.[37] Our kinetic data on the *S*-oxygenation are in keeping with the mechanism of the *N*-demethylation promoted by horseradish peroxidase in the generation of cation radical intermediate of the substrate.

Lewis and Ho carried out photochemical oxidation of *N*-methyl-*N*-methyl-d_3-*t*-butylamine and estimated the kinetic isotope effect (k_H/k_D) of 2.2 on the basis of the amount of CD_2O and CH_2O formed in the reaction.[38] The isotope effect, 2.2 they found in the photochemical oxidation of the tertiary amine, is believed to result from the deprotonation process going from the aminium radical to the α-amino alkyl radical. Meanwhile, in the enzymatic oxidation of tertiary amines (*N*-demethylation), kinetic isotope effects of similar magnitude, *i.e.*, 1.6—2.0 were observed by Miwa *et al.*[39] The isotope effects of a similar size in both chemical and enzymatic oxidations of tertiary amines suggest that one electron transfer process described above is also taking place in the enzymatic system. The enzymatic *N*-demethylation promoted by the rat liver microsomal cytochrome P-450 also involves the similar formation of an aminium radical intermediate prior to give an α-amino alkyl radical, which reacts subsequently with iron-bound "oxenoid" intermediate, eventually affording the *N*-monodemethylated amine and formaldehyde.

Alkyl ethers, such as anisole derivatives having a nucleophilic hetero atom, also have an isoelectronic structure of alkyl sulfides and tertiary amines. However, since the ether-oxygen is highly electronegative, one-electron transfer from the ether oxygen cannot be so facile unlike tertiary amines or sulfides. Therefore a process involving direct α-hydrogen abstraction and subsequent hydroxylation as shown in Eq. 2 (a) is more conceivable.

$$\mathrm{R\text{-}\ddot{S}\text{-}R'}, \qquad \mathrm{R\text{-}\overset{R''}{\underset{|}{\ddot{N}}}\text{-}R'}, \qquad \mathrm{R\text{-}\ddot{O}\text{-}R'}$$

In order to clarify the oxidation pathway of *O*-demethylation with the enzymatic system of cytochrome P-450, a kinetic study of the *O*-demethylation of *p*-substituted anisoles (**3a**—**e**) was performed.

Unlike the enzymatic oxygenation of sulfides, there

was no correlation between the rates of oxidation of NADPH and one electron oxidation potentials of anisoles. The effect of *p*-substituent in anisoles (**3**) on one electron oxidation potential is higher than that of thioanisoles (**1**); namely, the difference between the oxidation potentials of *p*-nitroanisole (**3e**) and *p*-methoxyanisole (**3a**) is 0.89 (V *vs.* SCE) while it is 0.59 (V *vs.* SCE) between those of thioanisole derivatives (**1a**) and (**1e**) (Table 2 and Fig. 2).

These results strongly suggest that the cation radical mechanism in Eq. 2 (b) is not acceptable.

In the electrochemical oxidation of several benzyl ethers, carried out by Mayeda *et al*,[40] however, they postulated a mechanistic pathway containing a cation radical intermediate (Eq. 10).

$$\ddot{\text{R}}\text{-O-CH}_2\text{Ph} \xrightarrow{-e^-} \text{R-}\overset{+\cdot}{\text{O}}\text{-CH}_2\text{Ph} \xrightarrow[\text{ii) } -e^-]{\text{i) } -H^+} \text{R-}\ddot{\text{O}}\text{-}\overset{+}{\text{C}}\text{HPh} \xrightarrow{H_2O} \text{R-O-}\underset{\text{OH}}{\text{CHPh}} \longrightarrow \text{R-OH} + \text{OHCPh} \quad (10)$$

Recently, the mechanism of the anodic cleavage of benzyl ethers was examined by Boyd *et al.*[41] using benzyl benzyl-α,α-d_2 ether. When it was oxidized by electrochemically, an intramolecular primary kinetic isotope effect of 1.9 was observed. This kinetic isotope effect was interpreted to be due to the deprotonation process *via* the cation radical intermediate of the ether (Eq. 10).

In the enzymatic oxygenation of several substrates leading to the hydroxylation and/or *O*-demethylation, small kinetic isotope effects were observed.[4,16-20] For example, in the *O*-demethylation of *p*-chloroanisoles (**3d**, **3d**-d_3) the value of 1.1 for k_H/k_D was obtained (Table 4). Generally the observed intermolecular isotope effect was small.

If one electron transfer from the oxygen atom of an ether to the iron-bound "oxenoid" intermediate of cytochrome P-450 takes place at the rate-determining step, the kinetic isotope effect would be around unity, when a mixture of *p*-dimethoxybenzene (**3a**) and *p*-di(methoxy-d_3)benzene (**3a**-d_6) is aerobically incubated with a microsomal cytochrome P-450 system, since ethers **3a** and **3a**-d_6 would have roughly the same one electron oxidation potential. The subsequent deprotonation from the cation radical of ether (Eq. 2 (b)) would be fast and irreversible. Meanwhile a kinetic isotope effect of ≈ 2 is expected to be obtained in the internal competitive enzymatic oxygenation of *p*-methoxy-d_3-anisole (**3a**-d_3) as in the electrochemical oxidation of benzyl benzyl-α,α-d_2 ether.[41]

However, a large isotope effect of 5.1 was observed in the competitive *O*-demethylations of *p*-dimethoxybenzene (**3a**) and *p*-di(methoxy-d_3)benzene (**3a**-d_6), and the kinetic isotope effect of 3.4 was observed in the oxidative *O*-demethylation of *p*-methoxy-d_3-anisole (**3a**-d_3) with rabbit liver microsomes (Tables 5 and 6). Recently, even a large kinetic isotope effect of 10 was reported in the oxidative *O*-demethylation of ether (**3a**-d_3) with the rat liver microsomes by Foster *et al.*[5]

All these large values of isotope effects and the kinetic result found on the enzymatic *O*-demethylation of anisoles support the hydroxylation to involve initial α-hydrogen abstraction and subsequent hydroxylation as illustrated in Eq. 2 (a), although we cannot rule out completely an alternative mechanism involving a direct insertion of active oxygen (oxene) into methoxy C–H bond.[42]

Experimental

Reagents. The following compounds were obtained from the commercial sources as indicated below; NADPH, $NADP^+$, D-glucose 6-phosphate (G6P), and D-glucose 6-phosphate dehydrogenase (G6P-DH), Oriental Yeast. *p*-Substituted thiophenols, methyl iodide, dimethyl disulfide, and triethylbenzylammonium chloride, Wako Chemicals. *p*-Substituted phenols and *p*-substituted anisoles, Tokyo Chemicals Co. Ltd. Methyl-d_3 iodide (min. 99.5%), Merck.

Organic Synthesis. *p*-Methyl- and *p*-chlorothioanisole (**1b**, **1d**) were obtained by treating *p*-methyl- and *p*-chlorothiophenol with methyl iodide in the presence of sodium hydroxide in good yield,[43,44] and isolated upon SiO_2-column chromatography (Merck Kieselgel 60, benzene as an eluent). *p*-Methoxythioanisole (**1a**) was prepared by the reaction of anisole and *S*-methyl methanethiosulfinate as described in a recent work by Oae *et al.*[45] *p*-Nitrothioanisole (**1e**) was synthesized by treatment of *p*-nitroaniline and dimethyl disulfide with *t*-butyl thionitrate.[46] Both **1a** and **1e** were purified through SiO_2-column chromatography. ^{1}H NMR (δ-values in $CDCl_3$, ppm); *p*-$MeOC_6H_4$–SMe,[45] 2.4 (3H, s), 3.75 (3H, s), 6.86 (2H, d J=9.0), 7.29 (2H, d J=9.0). *p*-MeC_6H_4–SMe,[47] 2.28 (3H, s), 2.41 (3H, s), 7.12 (4H, brs). *p*-ClC_6H_4–SMe,[48] 2.41 (3H, s), 7.03 (2H, d J=8.7), 7.12 (2H, d J=8.7). *p*-$NO_2C_6H_4$–SMe,[46] 2.55 (3H, s), 7.25 (2H, d J=9.4), 8.15 (2H, d J=9.4).

All the sulfoxides (**2**) used as authentic samples were synthesized by oxidation of the corresponding sulfides (**1**) with hydrogen peroxide[49] (in acetic acid) or *m*-chloroperbenzoic acid[50] (in dichloromethane), and were isolated through Al_2O_3-column chromatography (Wako, Alumina activated 200 mesh, AcOEt:$CHCl_3$=4:1 as eluent). ^{1}H NMR (δ-values in $CDCl_3$); *p*-$MeOC_6H_4$–$\underset{\downarrow}{\underset{O}{S}}$Me,[51] 2.70 (3H, s), 3.85 (3H, s), 7.01 (2H, d J=9.0), 7.60 (2H, d J=9.0). *p*-MeC_6H_4–$\underset{\downarrow}{\underset{O}{S}}$Me,[51] 2.4 (3H, s), 7.27 (2H, d J=8.6), 7.54 (2H, d J=8.6). C_6H_5–$\underset{\downarrow}{\underset{O}{S}}$Me,[52] 2.64 (3H, s), 7.4—7.6 (5H, m). *p*-ClC_6H_4–$\underset{\downarrow}{\underset{O}{S}}$Me,[53] 2.64 (3H, s), 7.39 (2H, d J=8.9), 7.44 (2H, d J=8.9). *p*-$NO_2C_6H_4$–$\underset{\downarrow}{\underset{O}{S}}$Me,[54] 2.8 (3H, s), 7.84 (2H, d J=9.0), 8.40 (2H, d J=9.0).

All the deuterated anisoles (**3a**-d_3, **3a**-d_6, and **3d**-d_3) were prepared by the reaction of the corresponding phenols (**4**) with methyl-d_3 iodide (min. 99.5%) and sodium hydroxide using a phase transfer catalyst (triethylbenzylammonium chloride) described by McKillop.[55] ^{1}H NMR (δ-values in $CDCl_3$) and *m/e*; *p*-$CD_3OC_6H_4OCH_3$,[5] 3.80 (3H, s), 6.80 (4H, s). *m/e*, 141 (M^+, 100%), 138 (0%). *p*-$CD_3OC_6H_4$-OCD_3,[5] 6.80 (4H, s). *m/e*, 144 (M^+, 100%), 141, 138 (0%). *p*-$CD_3OC_6H_4Cl$, 6.76 (2H, d J=9.0), 7.18 (2H, d J=9.0). *m/e*, 147 (M^+, ^{37}Cl 35%), 145 (M^+, ^{35}Cl 100%), 144, 142 (0%).

All the substrates were distilled or recrystallized prior to use. ^{1}H NMR spectra described above were recorded on a Perkin Elmer Hitachi R-20 (60 MHz) and mass spectra were recorded on a Hitachi RMU-6MG Mass Spectrometer.

Preparation of Microsomes, Purified Cytochrome P-450 and NADPH-Cytochrome P-450 Reductase. The liver microsomes (cytochrome p-450, 2.45 nmol/mg protein) were obtained from male rabbits (2—3 kg) pretreated with sodium phenobarbital (50 mg/kg of body weight, each day for 5 d) according to the procedure reported earlier,[23] while the purified cytochrome P-450 was obtained by the method of Imai and Sato.[56] NADPH-cytochrome P-450 reductase was purified by the method of Iyanagi *et al.*[57]

Enzyme Assay. The standard reaction mixture for assaying the *S*-oxygenation and the *O*-demethylation activities were made to contain 76—250 μmol of the substrates, the hepatic microsomes (130 mg protein), the NADPH generating system [30 μmol of $NADP^+$, 300 μmol of G6P (2 Na salt) and 36 units of G6P-DH], and phosphate buffer (pH 7.4, 0.2 M) in a final volume of 20 ml. The reaction mixture without G6P-DH was incubated for 5 min at 36 °C with stirring to dissolve the substrates, prior to the addition of G6P-DH into the mixture. After incubating the reaction mixture for 20 min at 36 °C under air, the reaction was stopped by adding 10 ml of acetone and 0.5 M of trichloroacetic acid (to pH 3) (or 5 ml of 15% zinc sulfate and 5 ml of saturated barium hydroxide) into the mixture which was cooled for 10 min at 0 °C to precipitate the protein. After centrifugation (3000 min^{-1}) of the mixture for 15 min, the supernatant was separated, neutralized by addition of aqueous KOH solution, and then was extracted 4 times with chloroform. The chloroform layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The residue was diluted to 1 ml with acetone and analyzed by gas chromatography (GLC: Hitachi 163 Gas Chromatography, 1 m glass column, 10% SE-30) and thin layer chromatography (TLC: Merck, Kieselgel 60-GF_{254}, and Aluminiumoxid GF-254, 60/E). The oxidation products were isolated by preparative TLC (chloroform was used as an eluent), and identified by comparison with authentic samples. Formaldehyde was measured by the Nash reagent.[30]

Kinetic Conditions of Oxidation of Both Sulfides and Ethers with Reconstituted System Containing Purified Cytochrome P-450. The reconstituted system (1.1 nmol of cytochrome P-*450* and 1.7 nmol of NADPH-cytochrome P-450 reductase) was allowed to stand at room temperature for 10 min in UV micro-cell, and then was diluted by 0.1 M phosphate buffer (pH 7.7) containing 100 nmol of NADPH and 6.6×10^{-3}% of detergent (Emalgen 913; KAO-ATLAS Chemicals) to 1 ml. After measuring the natural consumption of NADPH by following the absorbance at 340 nm due to NADPH, a methanolic solution of the substrate (2—30 μl, 20 mM) was added into the UV micro-cell and the mixture was aerobically incubated to initiate the reaction. The rate of oxygenation of the sulfide or ether was monitored at several time intervals by following the consumption of NADPH.

Measurement of the Difference Spectra Caused by Addition of Substrates to the Purified Cytochrome P-450. Cytochrome P-450 (1.1—10 μM in 0.2 M of phosphate buffer containing 20% glycerol) was placed into a 10 mm cuvette for spectrometric recording. The substrate was dissolved in methanol (0.5—30 μl), into which was added the sample solution. The same amount of methanol was placed in the reference cuvette for spectroscopic comparison.

Analytic Procedure. The amount of protein in the microsomes was determined by Lowry's method,[58] using bovin serum albumin. The concentration of cytochrome P-450 was determined by comparison with the carbon monoxide-difference spectrum of the dithionite-treated liver microsomes. The absorbance between 450 and 490 nm ($\Delta A_{450-490}$) was used for calculation of cytochrome P-450 content (ε, 91 $mM^{-1}\,cm^{-1}$).[59,60] The concentration of the purified cytochrome P-450 was determined by measuring the absorption of the oxidized form ($\Delta A_{418-500}$), which has a molar extinction coefficient of 107 $mM^{-1}\,cm^{-1}$.[56] (UV: JASCO UVIDEC-1 and Hitachi Model 200-20 Spectrophotometer).

The ratio of *p*-methoxyphenol (**4a**) to *p*-methoxy-d_3-phenol (**4a**-d_3) was calculated from the intensities of methoxyl and aromatic ring protons which were recorded on ^{1}H-FT-NMR spectroscopy. (JEOL FX-100). ^{1}H NMR (δ-values in $CDCl_3$); *p*-$CH_3OC_6H_4OH$, 3.76 (3H, s), 6.78 (4H, s). *p*-$CD_3OC_6H_4OH$, 6.78 (4H, s).

Measurement of Cyclic Voltammograms. Cyclic voltammograms of substrates were obtained in 0.1 M *n*-Bu_4NClO_4/CH_3CN. (scan rate; 300, 150, and 50 mV/s) (HOKUTO DENKO Ltd., POTENTIOSTAT/GALVANOSTAT HA-301)

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