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Enzymatic Oxidation of Alkyl Sulfides by Cytochrome P-450 and Hydroxyl Radical

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The oxidation of alkyl sulfides, sulfoxides, and sulfones was examined with either rabbit liver microsomes or a reconstituted system containing purified cytochrome P-450, *S*-dealkylation being found to take place more readily with alkyl sulfides bearing a higher acidic α-hydrogen. Similar results were obtained in the oxidation of alkyl sulfides by hydroxyl radical. Both *S*-dealkylation and *S*-oxygenation products are presumed to be formed *via* the cation radical intermediate.

N- and *O*-Dealkylation with hepatic microsomes and the reconstituted system with purified cytochrome P-450 has been studied with many substrates.[1] However, *S*-dealkylation with hepatic microsomes was observed only with a few anticancer agents such as 6-(methylthio)-purine and its analogues by Mazel *et al.*[2-4] who focused their attention on the effect of inhibitors and the dependency of inducers on *S*-dealkylase activity. They observed the formation of formaldehyde but not that of other products. 6-(Methylthio)purine was reported to be metabolized *in vivo* in man giving 6-mercaptopurine as a urinary metabolite.[5] However, the product analysis was incomplete, and the general nature of *S*-dealkylation has remained unexplored. We found a markedly large structural dependency of alkyl sulfide in the *S*-dealkylation with a few biomimetic oxygenase systems.[6,7]

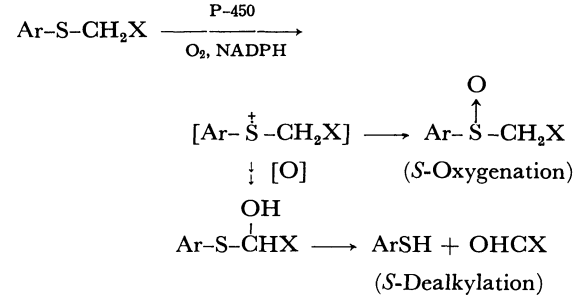
The enzymatic hydroxylation of hydrocarbons by cytochrome P-450 which has recently been examined,[8,9] is suggested to proceed *via* homolytic process involving hydrogen abstruction by iron-bound "oxenoid" species such as $(P\text{-}450\cdot FeO)^{3+}$ analogous to Compound I of peroxidase.[10,11] On the other hand, the oxygenation of sulfur atom of cyclic and acyclic sulfides by cytochrome P-450 seems to result by the electrophilic attack of active oxygen bound to the active site of the heme iron,[12] as suggested by Ullrich and Duppel.[13] We have observed that neither singlet oxygen ($^1O_2$) nor hydrogen peroxide is the active oxygenating species in view of the lack of inhibition with both DABCO (1,4-diazabicyclo[2.2.2]octane, inhibitor for singlet oxygen), and catalase in the reaction with the reconstituted system.[12]

Chemical oxygenation using model systems has been studied,[14-17] the best-studied being Fenton's reagent (hydroxyl radical) which catalyzes the hydroxylation of a variety of substrates.[17]

Gilbert *et al.*[18] and others[19,20] examined the reaction of alkyl sulfides with hydroxyl radical generated by treating hydrogen peroxide with transition metal ions of low oxidation state such as Fe(II) and Ti(III) by means of UV and ESR spectroscopic studies, and found that the cation radical intermediate (III) is formed as the key intermediate in the reaction of sulfides with hydroxyl radical.

We have extended our study on the oxygenation of various sulfides, sulfoxides, and sulfones with phenobarbital-induced rabbit liver microsomes and also with a reconstituted system of purified cytochrome P-450 in order to understand the whole mechanistic feature of the enzymatic oxygenation of organosulfur compounds. We found that both *S*-dealkylation and *S*-oxygenation take place simultaneously, the acidity of α-hydrogen of alkyl sulfides playing an important role in determining the mode of the enzymatic oxygenation (Scheme 1).

We also carried out the oxidation of sulfides by hydroxyl radical and found that a similar mode of oxidation to that in the enzymatic oxygenation takes place.



Scheme 1.

Experimental

Reagents. The following commercial compounds were used. $NADP^+$, D-glucose 6-phosphate (G6P), and D-glucose 6-phosphate dehydrogenase (G6P-DH) (Oriental Yeast); 16% Titanium trichloride solution (Kanto Chemicals); 30% Hydrogen peroxide, diphenyl disulfide, thioanisole, and thiophenol (Wako Chemicals).

Organic Synthesis. All the sulfides except thioanisole, diphenyl disulfide, and 4-(*p*-chlorophenyl)thiane were prepared by treating thiophenol with alkyl halides in the presence of sodium hydroxide in good yields,[21] and isolated by SiO_2 column chromatography (Merck Kieselgel 60, benzene as eluent). ^1H NMR (δ-value in $CDCl_3$, ppm) $PhSCH_2COPh$[22]; 4.05 (2H, s), 7.2—7.6 (8H, m), 7.8—8.0 (2H, m): $PhSCH_2$-C_6H_4-NO_2-*p*[23]; 4.01 (2H, s), 7.06 (5H, brs), 7.23 (2H, d, J=8.0), 7.93 (2H, d, J=8.0): $PhSCH_2CN$[24]; 3.44 (2H, s), 7.3—7.5 (5H, m): $PhSCH_2Ph$;[25] 4.11 (2H, s), 7.25 (10H, brs).

Deuterated sulfide, $PhSCD_2COPh$, was synthesized by the following method. Twenty equivalent of deuterium oxide (Merck, min 99.75%) was placed into a pyridine solution containing 2 mmol of phenacyl phenyl sulfide (5 ml) under argon atmosphere. The mixture was stirred at room temperature for 2 d, acidified (pH≈3) by adding 4 M HCl at 0 °C, and then extracted three times with chloroform (60 ml). The chloroform layer was washed with 1 M HCl (20 ml) to

remove a small amount of residual pyridine and dried over anhydrous magnesium sulfate. Chloroform was evaporated under reduced pressure and the deuterium content of phenacyl phenyl sulfide was determined from the ^1H NMR spectrum (95%-D_2). 4-(*p*-Chlorophenyl)thiane was prepared by the procedure described by Johnson.[26] ^1H NMR ($CDCl_3$) 2.1 (4H, m), 2.4 (1H, m), 2.8 (4H, m), 7.10 (2H, d, J=8.25), 7.25 (2H, d, J=8.25). All the sulfoxides used for the reactions and authentic samples were synthesized by the oxidation of corresponding sulfides with hydrogen peroxide in acetic acid.[27] ^1H NMR ($CDCl_3$), $PhS(O)CH_2COPh$;[28] 4.23 (1H, d' J=14.3), 4.25 (1H, d, J=14.3), 8—7.4 (10H, m): $PhS(O)CH_2$-C_6H_4-NO_2-*p*;[23] 4.0 (1H, d, J=12.8), 4.16 (1H, d, J=12.8), 7.1 (2H, d, J=9.0), 7.4 (5H, brs), 8.05 (2H, d, J=9.0): $PhS(O)CH_2CN$;[24] 3.70 (1H, d, J=15), 3.88 (1H, d, J=15), 7.6 (5H, m): $PhS(O)CH_2Ph$;[29] 4.0 (2H, brs), 7.4—6.9 (10H, m): $PhS(O)CH_3$;[30] 2.68 (2H, brs), 7.3—7.8 (5H, m), *trans*- and *cis*-4-(*p*-Chlorophenyl)thiane 1-oxide were separated by recrystallization (AcOEt) prior to Al_2O_3 column chromatography[31] (Wako, Alumina activated 200 mesh, AcOEt: $CHCl_3$=4 : 1 as eluent): *trans* sulfoxide; 2.0 (4H, m), 2.7 (1H, m), 2.7 (2H, dt, J=3.0, 12.0), 3.36 (2H, dt, J=2.4, 12.0), 7.0 (2H, d, J=7.5), 7.22 (2H, d, J=7.5): *cis* sulfoxide; 1.8 (2H, m), 2.6 (5H, m), 3.1 (2H, m), 7.11 (2H, d, J=7.5), 7.23 (2H, d, J=7.5). Sulfones were prepared by the treatment of corresponding sulfoxides with hydrogen peroxide in acetic acid.[23] ^1H NMR ($CDCl_3$) $PhS(O_2)CH_2$-C_6H_4-NO_2-*p*;[23] 4.4 (2H, s), 7.3 (2H, d, J=9.0), 7.6 (5H, m), 8.13 (2H, d, J=9.0): $PhS(O_2)CH_3$;[34] 3.05 (3H, s), 7.5—7.7 (3H, m), 7.8—8.1 (2H, m).

All the substrates were distilled or recrystallized prior to use. ^1H NMR spectra described above were recorded on a Parkin Elmer Hitachi R 20 (60 MHz).

Preparation of Microsomes, Purified Cytochrome P-450, and NADPH-Cytochrome P-450 Reductase. The hepatic microsomes (cytochrome P-450, 2.54 nmol/mg protein) were obtained from male rabbit (2—3 kg) pretreated with sodium phenobarbital (50 mg/kg of body weight, each day for 5 d) according to the procedure reported,[12] the purified cytochrome P-450 being obtained by the method of Imai and Sato.[35] NADPH–cytochrome P-450 reductase was purified by the method of Iyanagi *et al.*[36]

Enzyme Assay. The standard reaction mixture for assaying the *S*-dealkylation and *S*-oxygenation activities was made to contain 150—300 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, 0.1 M) in a final volume of 20 ml. The reaction mixture, except for G6P-DH, was incubated for 10 min at 36 °C to dissolve the substrates, prior to the addition of G6P-DH to the mixture. After the reaction mixture had been incubated at 36 °C for 2 h in the air, the reaction was stopped by adding 10 ml of acetone and 0.5 M of trichloroacetic acid (to pH 3) to the mixture which was cooled at 0 °C for 10 min in order to precipitate protein. After centrifugation (3000 rpm) of the mixture for 15 min, the supernatant layer was separated, neutralized by adding aqueous KOH solution, and then extracted 4 times with chloroform (200 ml). The chloroform layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The residue was diluted with acetone and analyzed by gas chromatography (GLC: Hitachi 163 Gas Chromatograph, 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 eluent), and identified by comparison with authentic samples. Formaldehyde was determined by the Nash reagent.[37]

Oxidation of Sulfides with Reconstituted System Containing Purified Cytochrome P-450. After a mixture of 35 nmol of cytochrome P-450, 38 nmol of the reductase and 0.3 ml of 1% detergent (Emalgen 913, KAO-ATRAS Chemicals) had been kept standing at room temperature for 5 min, 134 nmol of catalase, 22 ml of phosphate buffer (pH 7.4, 0.1 M), 250 μmol of G6P, 5 μmol of $NADP^+$, and 36 units of G6P-DH were added to the mixture. The substrate (480 μmol) was then suspended into the reaction mixture and the whole solution was incubated at room temperature for 1 h in the air. After the usual work-up, the products obtained were analyzed by GLC.

Measurement of the Difference Spectra Caused by the Addition of Substrates to the Purified Cytochrome P-450. Cytochrome P-450 (3.6 μM in 0.1 M of phosphate buffer containing 20% glycerol) was placed in 10 mm cuvettes for spectrometric recording. The substrate was dissolved in methanol (0.5—30 μl), into which was added the sample solution. The same volume of methanol was placed in the reference cuvette.

Analytic Procedure. The amount of protein in the microsomes was determined by the method of Lowry *et al.*[38] using bovin serum albumine. The concentration of cytochrome P-450 was determined by the carbon monoxide-difference spectrum of 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 $mM^{-1}\,cm^{-1}$).[39,40] The concentration of 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 $mM^{-1}\,cm^{-1}$.[35] (UV: JASCO UVIDEC-1)

Oxidation of Sulfides by Iron(II) Perchlorate–Hydrogen Peroxide System. A mixture of sulfide (0.2 mmol) and iron(II) perchlorate (250 mg, 1 mmol) in methanol–7% perchloric acid aqueous solution (10 ml–5 ml) was stirred at room temperature under argon atomsphere for 5 min. 7% Perchloric acid (5 ml) containing 0.2 ml of hydrogen peroxide (30% solution in water) was then added dropwise slowly to the ethanolic solution and the reaction mixture was stirred for 10 min. The reaction mixture was extracted three times with chloroform (100 ml). The products were determined by GLC and SiO_2-TLC (eluent; $CHCl_3$).

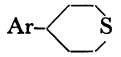
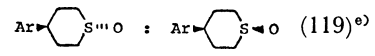
Oxidation of Sulfides by Titanium Trichloride–Hydrogen Peroxide System. A mixture of sulfide (0.25 mmol) and methanol (10 ml) was stirred at room temperature under argon atmosphere for 5 min. Titanium trichloride solution (16% solution, 0.5 ml) was added in one portion to the mixture which was stirred for 1 min prior to the addition of hydrogen peroxide (30%, 0.2 ml) dissolved in 5 ml of H_2O, and the reaction mixture was stirred further for 5 min. The reaction was stopped by adding 30 ml of water and the mixture was subjected to the usual work-up.

Preparation of ^{18}O-Labeled Hepatic Microsomes. The hepatic microsome obtained above was diluted with $H_2{}^{18}O$ which contains 1.5% excess ^{18}O while phosphate buffer was prepared with $H_2{}^{18}O$-containing 1.5% excess ^{18}O (The British Oxygen Company Limited).

Oxidation of Sulfides by ^{18}O-Labeled Titanium Trichloride–Hydrogen Peroxide System. Five ml of ^{18}O-labeled (1.5 atom % excess) water was used instead of ordinary water.

^{18}O-Analyses. All the ^{18}O-labeled substrates were placed in a breakable glass tube together with well-dried $HgCl_2$ and $Hg(CN)_2$. The tube was evacuated by diffusion pump for 15 min, and sealed. The sealed tube was heated at 500 °C for 12 h. The gas (CO_2) produced was distilled repeatedly 3 times and transferred into a sampler of the mass spectrometer (Hitachi RMU 6MG). The ^{18}O-content of carbon dioxide

Table 1. Oxidation of sulfides by microsomes and reconstituted cytochrome P-450

Material	(μmol)	System[a]	Products (nmol/nmol P-450)
1a	(290)	A	**2a** (100) **3a** (5.7)[b]
1b	(180)	A	**2b** (100)
1c	(240)	A	**2c** (78) **5** (44)[c]
		B	**2c** (33) **5** (4.0)
1d	(170)	A	**2d** (30) **3d** (30) **4d** (91)[d] **5** (60)
		B	**2d** (12) **4d** (25) **5** (4.3)
1e	(170)	A	**2e** (91) **4e** (4.7) **5** (100)
 **1f**	(153)	A	 (119)[e] **2f-*t*** **2f-*c*** 66 : 34

a) System A; microsomes, system B; reconstituted system. b) Formaldehyde was measured in a separate experiment by using the Nash reagent. c) Isolated yields for the sulfoxides and sulfones by SiO_2 or Al_2O_3 PTLC. d) Yields determined by GLC described in Experimental. e) *Trans-cis* ratio determined by GLC (OV-1, 1m glass column).

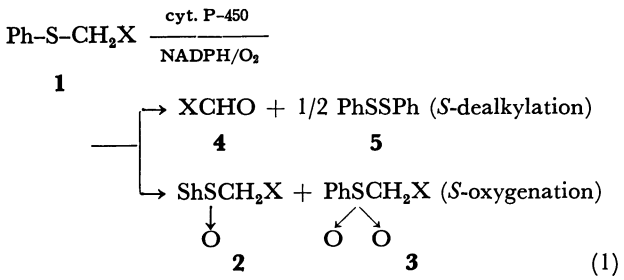
was calculated from the height of flat peaks of *m/e* 44 and 46.

Measurement of Cyclic Voltammogram. Cyclic voltammograms of substrates were obtained in 0.1 M n-Bu_4NClO_4/CH_3CN solution. (scan rate: 300, 150, and 50 mV/s) (Hokuto Denko Ltd., Potentiostat Galvanostat HA-301).

Results

When the β-keto sulfide (**1e**) was incubated with the hepatic microsomes, *S*-dealkylation and *S*-oxygenation took place simultaneously, giving aldehyde (**4e**) and diphenyl disulfide (**5**) as the *S*-dealkylated products and the corresponding sulfoxide (**2e**) as the *S*-oxygenated product (Table 1). Similarly, *p*-nitrobenzyl phenyl sulfide (**1d**) gave *p*-nitrobenzaldehyde (**4d**) together with a comparable amount of **5**, accompanied by sulfoxide (**2d**) and sulfone (**3d**) as the *S*-oxygenated products. This is in contrast to the chemical oxidation of the sulfides (**1d**, **1e**) with hydrogen peroxide or *m*-chloroperbenzoic acid which gave only sulfoxides (**2d**, **2e**). Compounds **2d** and **3d**, microsomal oxidation products, were separated by Al_2O_3-PTLC (eluent; $CHCl_3$: AcOEt=1 : 1) and identified by comparison of mass spectra and GLC with those of authentic samples.

In the oxidation of thioanisole (**1a**) and benzyl



(1)

X: **a** = H, **b** = Ph, **c** = CN, **d** = C_6H_4–NO_2-*p*, **e** = COPh

phenyl sulfide (**1b**), *S*-oxygenation was found to take place predominantly giving the corresponding sulfoxides (**2a** and **2b**), respectively.

Thus, in the microsomal oxidation of alkyl sulfides, the mode of the reaction appears to depend markedly on the structure of the alkyl group; *viz.*, the ratio between the *S*-dealkylation and the *S*-oxygenation was found to increase with the increase of the acidity of α-hydrogen of the alkyl sulfide. (Tables 1 and 8). This trend is analogous to that in the biomimetic oxidation of the same sulfides with both Co^{II}(bzacen)–O_2 system and Udenfriend's system.[6,7]

Table 2. Relative S-dealkylation and S-oxygenation activity of rabbit liver microsomes

Additions		Products[a] PhSCH$_2$COPh (O)	Products[a] PhSSPh
Complete system	Ms. + O_2 + NADPH generating system	100	100
Complete system	minus NADPH generating system	0	0
Complete system	minus Ms. plus boiled Ms	0	0
Complete system	plus 50% CO	80	71

a) Products were compared in final yields determined by GLC described in Experimental part.

The requirements for cofactors in the reaction of the β-keto sulfide (**1e**) are given in Table 2. NADPH was required in the enzymatic reaction while boiled microsomes completely lost activities for both *S*-dealkylation and *S*-oxygenation. Carbon monoxide also inhibited enzymatic activity for both *S*-dealkylation and *S*-oxygenation of **1e**, respectively. This indicates that both *S*-dealkylation and *S*-oxygenation reactions are typical oxidation reactions promoted by cytochrome P-450.

Cyanomethyl phenyl sulfide (**1c**) and *p*-nitrobenzyl phenyl sulfide (**1d**) were metabolized with a reconstituted system, containing purified cytochrome P-450, NADPH-cytochrome P-450 reductase, 1% detergent and a NADPH generating system, and retaining similar activities for both *S*-dealkylation and *S*-oxygenation.

The sulfide (**1d**), however, was insoluble in a reconstituted system so that the net activity was low, and yet the oxidation of **1d** with the reconstituted cytochrome P-450 system was found to proceed in the two types of oxidation as denoted by cytochrome P-450.

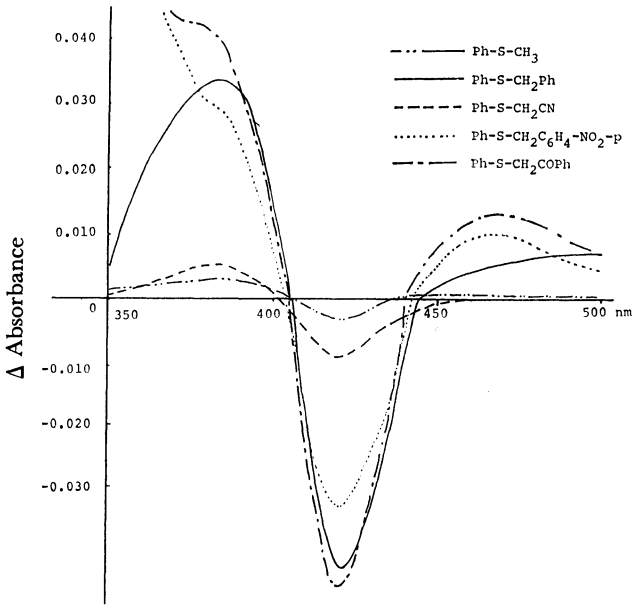
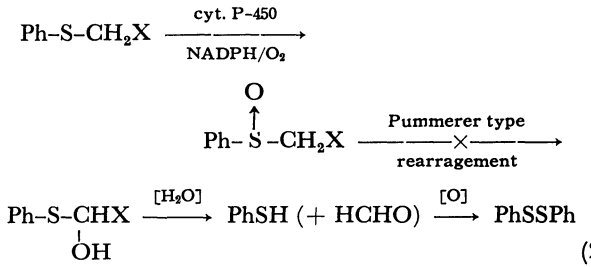


Fig. 1. Cytochrome P-450 from phenobarbital-treated rabbit were diluted to 3.6 μmol in 0.1 M phosphate buffer. After establishment of a base line of equal light absorbance, methanolic solution of sulfide (0.2 mM) was added to the sample cuvvett.

When methanolic solutions (0.5—30 μl) of these sulfides (**1a—e**) were added to the buffer solution of purified cytochrome P-450 (2 ml), and the difference spectra with iron(III) heme were found to be typical type I,[41,42] having a maximum at 385—390 nm and a minimum at 420 nm (Fig. 1), suggesting that the mode of the whole interaction between the sulfides and the hemoprotein is similar during the course of enzymatic oxidation.

The microsomal oxidation of sulfoxides (**2a, d**) and sulfones (**3a, d**) was performed for further assay of both *S*-oxygenation and *S*-dealkylation (Table 3). However, neither sulfoxides nor sulfones, both of which possess higher acidic α-hydrogen than the corresponding sulfides (see Table 8), underwent facile *S*-dealkylation, *e.g.*, *p*-nitrobenzyl phenyl sulfoxide (**2d**) gave both *S*-dealkylated product (**4d**) and *S*-oxygenated product (**3d**). However, the reaction was slow and the product ratio between the former and the latter is *ca.* 1/3 while in the oxygenation of the corresponding sulfide (**1d**) the ratio is *ca.* 4/3 (Table 3).

In the reaction of the sulfoxide to sulfone with microsomal cytochrome P-450, diphenyl disulfide (**5**) was not obtained as *S*-dealkylated product, despite the formation of the aldehyde, ruling out the possibility of the normal Pummerer rearrangement involving sulfoxide as the reaction intermediate for the *S*-dealkylation of sulfide.



(2)

Thioanisole (**1a**) and benzyl phenyl sulfide (**1b**) were found to react with hydroxyl radical generated by the reaction of iron(II) or titanium(III)–hydrogen peroxide system, giving only *S*-oxygenated products (**2a** and **2b**) in contrast to the oxidation of (**1c—e**), which gave both *S*-oxygenated products (**2c—e**) and *S*-dealkylated product (**4**) (Tables 4 and 5). The ratio of **5**/**2** increased from **1c** to **1e**. This is in line with the results given in both the microsomal and the purified cytochrome P-450 oxidations of alkyl sulfides (Table 1). The ratio between *S*-dealkylation and *S*-oxygenation

TABLE 3. MICROSOMAL OXIDATION OF SULFIDES, SULFOXIDES, AND SULFONES

Material (μmol)		Products (nmol/nmol P-450)[a]	Recovered (%)
1a	(290)	**2a** (100) **4a** (5.7)[b]	67
2a	(330)	**3a** (182) **4a** (trace)	64
3a	(280)	**4a** (trace)	95
1d	(170)	**2d** (30) **3d** (30) **4d** (91)[c]	75
		5 (60)	75
2d	(180)	**3d** (38) **4d** (14)[c]	89
3d	(150)	**4d** (2.8)[c]	96

a) Isolated yields by SiO_2 or Al_2O_3 PTLC. b) Determined in a separate experiment by using the Nash reagent. c) Determined by GLC as described in Experimental.

TABLE 4. THE REACTION OF SULFIDES WITH HYDROXYL RADICAL GENERATED FROM IRON(II) PERCHLORATE–HYDROGEN PEROXIDE SYSTEM

Substrate[a]	System[b]	Products (μmol)[c]
1a	A	**2a** (14)
1b	A	**2b** (9.3)
	B	**2b** (13)
	C	**2b** (14)
1c	A	**2c** (7.5) **5** (0.23)
	B	**2c** (6.7) **5** (0.8)
	C	**2c** (8.2) **5** (1.1)
1d	A	**2d** (8.0) **5** (2.8)
	B	**2d** (21) **5** (3.7)
	C	**2d** (10) **5** (4.7)
1e	A	**2e** (4.6) **5** (4.8)
	B	**2e** (24) **5** (4.6)
	C	**2e** (8.2) **5** (15)
1f[d]	A	**2f**-*t* : **2f**-*c* (87)[e] 80 : 20

a) 0.2 mmol of sulfides were used in all cases. b) System A; see Experimental. System B; H_2O was used instead of 7% perchloric acid aq solution. System C; buffer (pH= 7.0) was used instead of 7% perchloric acid solution. c) Determined by GLC and SiO_2 TLC. d) 212 mg (1.2 mmol) of sulfide used. e) Determined by GLC (10% OV-1, 1m glass column).

Table 5. Reaction of sulfides with hydroxyl radical generated from titanium trichloride–hydrogen peroxide system

Substrate[a)]	Products (μmol)[b)]	
1a		**2a** (250)
1b		**2b** (247)
1c	**2c** (226)	**5** (1.0)
1d	**2d** (109)	**5** (2.0)
1e	**2e** (57)	**5** (8.5)

a) 250 μmol of sulfides was suspended in all cases.
b) Determined by GLC and SiO_2 TLC.

was found to vary in different media (Table 4, systems A, B, and C), while the tendency for *S*-dealkylation remained unchanged. The difference between the microsomal and the reconstituted systems can be explained on the basis of varying conditions of media used.

When the 95% deuterated sulfide (**1e**-d_2, $PhSCD_2COPh$) was incubated with hepatic microsomes in the complete system at 36 °C, both *S*-dealkylation and S-oxygenation proceeded concurrently. In this reaction only a small kinetic isotope effect ($k_H/k_D \fallingdotseq 1.2$) was observed while the ratio **5**/**2** remained nearly the same with both **1e** and **1e**-d_2. The k_H/k_D values were computed approximately by [**5**/**2**]/[**5**-d_2/**2**-d_2] for both microsomal and Fenton's oxidation. In the reaction with Fenton's system (system A), k_H/k_D was 1.3, being analogous to that in the oxidation (Table 6). The deuterium label of the recovered sulfide (**1e**-d_2) remained in both cases (measured by ^1H NMR).

When the oxidation of sulfide (**1c**) by both the microsomal and Fenton's oxidation systems was carried out in ^{18}O-labeled phosphate buffer solution, only 1.3 and 4.7% of oxygen were found to be incorporated into the sulfoxide (**2c**) from heavy water, $H_2{}^{18}O$ used (Table 7).

Table 6. Kinetic isotope effect for the oxidation of phenacyl phenyl sulfide by both rabbit liver microsomes and iron(II)–hydrogen peroxide system

System	Substrate $PhSCH_2COPh$		$PhSCD_2COPh$		k_H/k_D[a)]
	products[b)] **2e**	**5**	**2e**-d_2	**5**-d_2	
Microsomes	29	32	27	24	1.2
Fe(II)–H_2O_2	4.6	4.8	3.9	3.2	1.3

a) Estimated roughly by [**5**/**2e**]/[**5**-d_2/**2e**-d_2]. b) Yields measured by GLC (μmol).

Table 7. ^{18}O-Contents of phenyl methyl sulfoxide produced by both liver microsomes and Fenton's system

	$H_2{}^{18}O$ (A) used (%)	$Ph\overset{^{18}O\uparrow}{S}CH_3$ (B) (%)	O-content from water (B/A×100) (%)
Liver microsomes	1.05[a)]	0.014[a)]	1.3
$TiCl_3$–H_2O_2	1.32[b)]	0.062[a)]	4.7

a) Observed values. b) Calculated values (see Experimental).

Discussion

A multienzyme system containing cytochrome P-450 located in liver microsomes, and a reconstituted system of the purified cytochrome P-450 and its reductase from liver microsomes of phenobarbital-treated rabbit and NADPH were found to catalyze readily both *S*-dealkylation and *S*-oxygenation of common sulfides and only sluggishly those of sulfoxides and sulfones.

The relatively higher acidity of the α-hydrogen is required for *S*-dealkylation, though other factors are also necessary, since sulfoxides (**2**) and sulfones (**3**) having more acidic α-hydrogen are less reactive than the sulfides for *S*-dealkylation (Tables 1 and 8).

Free radicals are stabilized substantially when the central carbon is substituted by a divalent sulfur atom but less stabilized by an adjacent α-sulfinyl or α-sulfonyl group.[43)] This is in line with the trend of the Q-values of substituted vinyl compounds. Vinyl sulfoxide possesses the smallest Q-value in copolymerization as suggested by the stability of α-substituted free radicals ($RS\dot{C}HR' > RS(O_2)\dot{C}HR' \geq RS(O)\dot{C}HR'$ in Table 8). Cyano, *p*-nitrophenyl, and carbonyl groups have larger Q-values than benzyl, and methyl groups.[44)] Such a free radical-stabilizing effect of sulfenyl group may be partially responsible for the facile *S*-dealkylation of sulfides.

Several substrates, which are deuterated prior to hydroxylation at aliphatic sites have been tested with hepatic microsomes. Groves *et al.*[8)] suggested that hydroxylation by highly purified liver microsomal cytochrome P-450 involves the direct hydrogen abstraction by iron-bound "oxenoid" species $(P\text{-}450\cdot FeO)^{3+}$, which is analogous to Compound I of peroxidase,[11)] to generate the carbon radical intermediates on the basis of their observed kinetic isotope effects ($k_H/k_D = 11.5 \pm 1$)[8)] and $k_H/k_D = 11 \pm 1$ for benzylic hydroxylation of 1,3-diphenylpropane (and 1,1-dideuterio derivative) by Hjelmeland *et al.*[9)]

Sulfides are good substrates for the oxygenation with cytochrome P-450 enzyme system to give sulfoxides.[12,45)] Since the "oxenoid" intermediate should be highly electron deficient, *S*-oxygenation is expected to be initiated by an electron transfer from divalent sulfur atom to "oxenoid" species, generating sulfenium cation radical. The oxygenation of alkyl sulfides with a highly purified cytochrome P-450 gave kinetic evidence for one-electron transfer mechanism.[46)]

$$\text{Ar–S–CH}_3 \xrightarrow[\text{rate determining step}]{\text{cyt. P-450 (FeO)}^{3+}}$$

$$\text{Ar–}\overset{+}{\dot{\text{S}}}\text{–CH}_3 + (\text{FeO})^{2+} \longrightarrow \text{Ar–}\overset{\overset{\text{O}}{\uparrow}}{\text{S}}\text{–CH}_3 + \text{Fe(III)} \quad (3)$$

In the oxidative dealkylation of tertiary amine by enzymes, a similar cation radical intermediate seems to be involved.[47)] The oxygenation of aminopyrine by horseradish peroxidase has been suggested to involve the cation radical intermediate on the basis of ESR spectroscopic studies.[48)]

TABLE 8. pK_a-VALUES OF METHANE DERIVATIVES OF ELECTRON-WITHDRAWING GROUP AND THE Q-VALUES OF VINYL DERIVATIVES IN RADICAL COPOLYMERIZATION

Compound	pK_a	Compound	Q
$PhCO\underline{CH_3}$[a]	24.7	$CH_2=CHCOPh$[b]	1.4
$NC\underline{CH_3}$[a]	31.3	$CH_2=CH-C_6H_4-NO_2-p$[b]	1.63
$Ph\underline{CH_3}$[c]	40.9	$CH_2=CHCN$[b]	1.78
$CH_3-\underline{H}$[c]	48	$CH_2=CHPh$[b]	1.0
$CH_3S(O)\underline{CH_3}$[c]	33	$CH_2=CH_2$[b]	0.015
$CH_3S(O_2)\underline{CH_3}$[c]	29	$CH_2=CHSCH_3$[d]	0.34
$PhS\underline{CH_3}$[a]	48	$CH_2=CHS(O)CH_3$[d]	0.06—0.01
$PhS(O_2)\underline{CH_3}$[a]	29	$CH_2=CHS(O_2)CH_3$[a]	0.11

a) E. Block, "Reactions of Organosulfur Compounds," Academic Press, New York (1978). b) "Kyojugo," ed by Kobunshi-gakkai, Baifukan, Tokyo (1975), Vol. 1, p. 396. c) T.H. Lowry and K.S. Richardson, "Mechanism and Theory in Organic Chemistry," Harper and Row Publishers, New York (1976). d) W. Tagaki, "Organic Chemistry of Sulfur," ed by S. Oae, Plenum Press, New York (1977), p.231.

$$\begin{aligned} &R_2N-CH_2R \xrightarrow{-e^-} R_2\overset{+}{N}-CH_2R \xrightarrow{-H^+} \\ &\qquad\qquad \downarrow (\text{or } -H\cdot) \\ &R_2N-\dot{C}HR \xrightarrow{-e^-} R_2\overset{+}{N}=CHR \xrightarrow{H_2O} \\ &[R_2N-\underset{\underset{OH}{|}}{CHR}] \longrightarrow R_2NH + RCHO \end{aligned} \qquad (4)$$

α-Hydroxyl sulfides (III) are formed as the intermediates *via* initial electron-transfer, subsequent proton removal, and final hydroxylation in the *S*-dealkylation (Eq. 5), and the formation of the α-sulfenyl radical (II) by direct hydrogen abstraction by electron deficient $(FeO)^{3+}$ is unlikely, since the ease of the *S*-dealkylation was found to be correlated not with the order of radical-stabilizing effect of α-substituent but with the acidity of α-hydrogen of the alkyl sulfides used.[49,50]

$$R-S-CH_2R' \xrightarrow[NADPH/O_2]{\text{cyt. P-450}} \underset{(III)}{R-S-\underset{\underset{OH}{|}}{CHR'}} \xrightarrow{H_2O} RSH + R'CHO \qquad (5)$$

The kinetic isotope effect observed for the *S*-dealkylation of phenacyl phenyl sulfide (**1e**) was rather small ($k_H/k_D=1.2$), differing from the results obtained by Groves *et al.*[8] and Hjelmeland *et al.*[9] A similar trend was observed in the reaction of sulfides with hydroxyl radical (Table 6).

Hydroxyl radical is an electrophilic species, reacting readily with electron rich sulfenyl sulfur, and giving the corresponding sulfenium cation radicals.

$$R-S-R' + \cdot OH \longrightarrow R-\overset{+}{S}-R + OH^- \qquad (6)$$

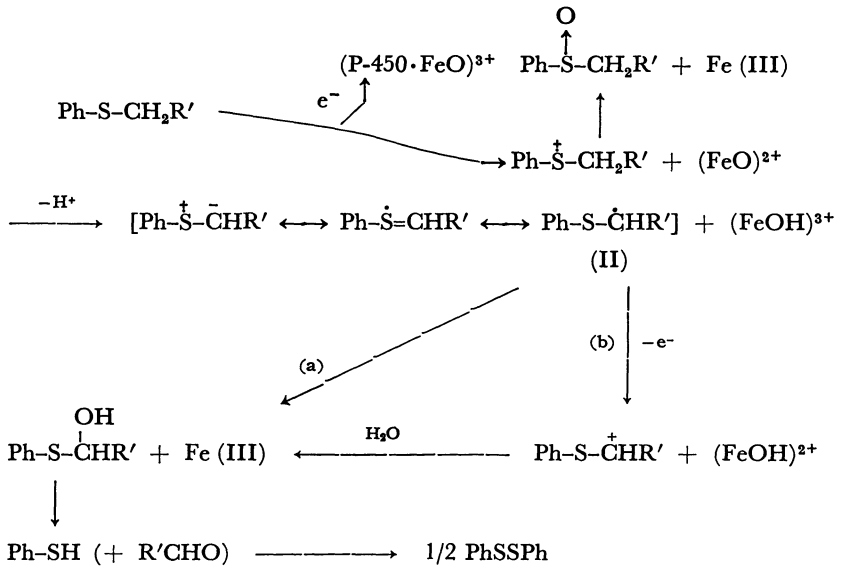
The reaction of sulfides with hydroxyl radical has been studied extensively by Gilbert *et al.*[18] and others[19,20] by means of UV and ESR and is illustrated in the following scheme (Scheme 2).

In this Scheme, step 4 should definitely be accelerated by either an electron-withdrawing substituents R′ or a group which stabilizes the intermediate radical (II), or by both. The Scheme is in line with our results with Fenton's oxidation system. In many ways, the reaction of step 4 can be considered as a 1,2 elimination of Elcb type,[51] and somewhat similar to the Pummerer rearrangement which shows a secondary kinetic isotope effect.[52] In the oxidation of tertiary amines, the deprotonation (Eq. 4) from nitrogen cation radical intermediate gives a kinetic isotope effect ($k_H/k_D=2.2$).[53]

Observation on the *S*-dealkylation can be interpreted, if we assume that the sulfenium cation radical initially formed is the common intermediate for both *S*-dealkylation and *S*-oxygenation through one electron transfer from divalent sulfur atom to the highly electron deficient "oxenoid." Electron-withdrawing substituent R′ would

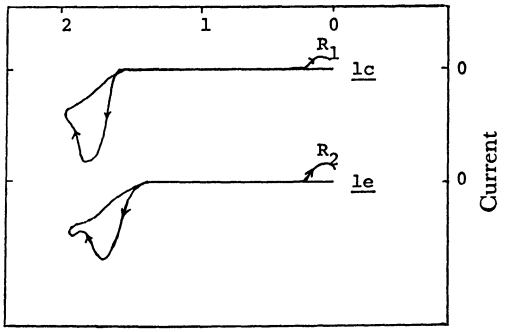
$$\begin{aligned} &R-S-CH_2R' + \cdot OH \xrightarrow[\text{controlled step}]{\text{diffusion}} \underset{\mathbf{A}}{R-\overset{OH}{\overset{|}{\dot{S}}}-CH_2R'} \\ &\mathbf{A} \xrightarrow[\text{low sulfide conc. } (<10^{-4}\,M)]{\text{fast } -H_2O} R-\dot{S}=CHR' \longleftrightarrow \underset{(II)}{R-S-\dot{C}HR'} \\ &\quad \Big| \text{ step 1} \qquad\qquad -H^+ \nearrow \text{ step 4} \\ &\text{high conc. } (>10^{-4}\,M) \\ &\quad \downarrow \\ &\underset{\text{short lived}}{\underset{R-\dot{S}-CH_2R'}{\overset{OH}{\overset{|}{R-S-CH_2R'}}}} \xrightarrow[-H_2O \text{ step 2}]{H^+} \left(\begin{matrix} R-S-CH_2R' \\ \| \\ R-S-CH_2R' \end{matrix}\right)^{+} \underset{\text{step 3}}{\rightleftharpoons} R-S-CH_2R' + R-\overset{+\cdot}{S}-CH_2R' \end{aligned}$$

Scheme 2.



Scheme 3.

facilitate the α-proton removal to form the α-sulfenyl radical (II) which upon subsequent hydroxylation would undergo facile hydrolysis to afford the aldehyde and thiophenol which is readily oxidized further to diphenyl disulfide (Scheme 3). The cyclic voltammograms of sulfides (Fig. 2) show the ready α-proton removal from the sulfenium cation radicals as suggested by Sato and Kamada.[56] However, we cannot rule out the possibility of path (b) in Scheme 3.



Potential (V *vs.* SCE)

$$PhSCH_2R' \xrightarrow{-e^-} PhS\dot{C}H_2R' \xrightarrow{-H^+} PhS\dot{C}HR'$$

Fig. 2. Cyclic voltammograms for sulfides in CH_3CN *vs.* SCE. Cathodic peaks (R_1 and R_2) suggest the abstraction of a proton.[56]

One way to demonstrate the generation of the sulfenium cation radical as the reaction intermediate by chemical procedure is to examine whether or not the oxygen of the sulfoxide formed by both microsomal and Fenton's oxidation originates not only from the "oxenoid" bound to iron of heme protein but also from water. The reaction of the sulfenium cation radicals of sulfides with water has been examined somewhat in detail and shown by Evans and Blount[54] and Murata and Shine[55] to give the corresponding sulfoxides stoichiometrically.[7] However, in the reaction of thioanisole (**1a**) with Ti(III)–H_2O_2 system, the amount of ^{18}O incorporated into the sulfoxide (**2a**) was only

$$2\,R_2S^{\ddagger} + H_2O \longrightarrow R_2S{\rightarrow}O + R_2S + 2\,H^+ \qquad (7)$$

4.7% of $H_2{}^{18}O$ used. This indicates that the cation radical reacts with hydroxyl radical preferentially even though the cation radical is surrounded by water of the medium.

The incorporation of ^{18}O into the sulfoxide through the microsomal oxygenation was only 1.3% of $H_2{}^{18}O$ used. In the oxygenation of sulfides with cytochrome P-450, the substrate seems to be tightly coordinated to the active site; the amount of water around the reaction site of the enzyme is too small to compete with "oxenoid" bound to the enzymatic active site so that the ^{18}O incorporation would be smaller than that with Fenton's system. The slight incorporation of ^{18}O into the sulfoxide from $H_2{}^{18}O$ water used in the microsomal oxygenation suggests that the transient intermediate, presumably the sulfenium cation radical, is still tightly held in the cavity of the enzyme which lacks enough water and eventually reacts oxygen bound to the enzyme. The ^{18}O experiment gave no information whether the key active sulfur intermediate is the sulfenium cation radical or dication. However, involvement of the dication species is quite unlikely in view of our earlier work, in which *p*-substituted thioanisole derivatives were oxygenated by a reconstituted system of purified cytochrome P-450, V_{max}'s of oxygenation being correlated nicely with Hammett σ^+-values rather than σ-values ($\rho^+ = -0.16$), and also with one electron oxidation potential of sulfides.[46]

In this study, we found a large structural dependency of alkyl sulfide in the *S*-dealkylation with both microsomal and hydroxyl radical oxidation systems. The over-all reaction may be explained by the generation of cation radical species as the common intermediate for both *S*-dealkylation and *S*-oxygenation as suggested in the enzymatic *N*-dealkylation.

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