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Hydroxylation of Acetanilide by Iron-Sulfur and Nickel-Sulfur Complex Systems

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The hydroxylation of acetanilide by iron-sulfur complexes produces *p*- and *o*-acetaminophenols as main products. The yield of *p*-acetaminophenol is 34.8 and 31.4% in the catalytic systems of dithiothreitol(DTT)-iron-inorganic sulfur (S) and glutathione (GSH)-Fe-S complexes, respectively. The turnover number of the acetanilide-hydroxylation is 3.9×10^{-2} and 3.5×10^{-2} (mol *p*-acetaminophenol/min/mol complex) in the DTT-Fe-S and GSH-Fe-S complex systems, respectively. The DTT-nickel complex incorporates inorganic sulfur to form unstable DTT-Ni-S complex. The DTT-Ni complexes have also high catalytic effect on the hydroxylation of acetanilide.

Keywords——acetanilide hydroxylation; iron-sulfur complex catalyst; nickel-sulfur complex catalyst; iron-sulfur protein model; drug metabolism

Iron-sulfur proteins function as electron carriers in biologically important reactions such as photosynthesis, nitrogen fixation, and steroid hydroxylation. The structural models of iron-sulfur proteins have been extensively investigated.[2] We have also studied the incorporation of inorganic sulfur into model iron-sulfur complexes to clarify the nature and function of the inorganic sulfur.[3] However, little studies on catalytic model systems for iron-sulfur proteins have been carried out. Iron-sulfur protein and cytochrome P-450 together participate in steroid 11β-hydroxylation of adrenal cortex and in camphor hydroxylation of microorganism. In addition, it has been recently reported that iron-sulfur protein from Pseudomonas putida acts as a terminal enzyme in 4-methoxybenzoate monooxygenase reaction.[4] The catalytic activity of iron-sulfur proteins has attracted the attention of us, and we have previously found that model iron-sulfur compounds catalyze hydroxylation of aniline.[5] This paper deals with the hydroxylation of acetanilide by iron-sulfur and nickel-sulfur complexes.

Experimental

Dithiothreitol (DTT), glutathione (GSH), acetanilide, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma. ^{14}C-Acetanilide (2.13 mCi/mM) was purchased from the Radiochemical Centre. Other chemical materials were commercially available reagent grade. Model iron-sulfur complexes were prepared by mixing the sulfur-containing ligands, sodium sulfide (or sodium selenide), and ferric chloride in an aqueous solution at pH 9.0, and were characterized by visible absorption and electron spin resonance (ESR) spectra.[5] Solutions of ferric chloride and sodium sulfide were freshly prepared with deionized water and were standerdized with ethylenediaminetetracetic acid (EDTA) and iodine respectively. It has been found that the molar ratio of sulfide (or selenide) to iron in the model complexes is 1:1 and that the stability of the complexes decreases in the order, DTT-Fe-S$\geq$DTT-Fe-Se$\gg$DTT-Fe.[6] Nickel complex of DTT was prepared by mixing the ligand and nickel chloride in an aqueous solution at pH 9.0, and was characterized spectrophotometrically.

1) Location: *Yoshida, Shimoadachi-cho, Sakyo-ku, Kyoto, 606, Japan.*
2) *a*) J.C.M. Tsibris and R.H. Woody, *Coord. Chem. Rev.*, **5**, 417 (1970); *b*) L. Qur, Jr., J.R. Anglin, M.A. Bobrik, A. Davison, and R.H. Holm, *J. Am. Chem. Soc.*, **96**, 6042 (1974).
3) *a*) Y. Sugiura and H. Tanaka, *Biochem. Biophys. Res. Commun.*, **46**, 335 (1972); *b*) Y. Sugiura, M. Kunishima, and H. Tanaka, *Biochem. Biophys. Res. Commun.*, **49**, 1518 (1972); *c*) Y. Sugiura, M. Kunishima, H. Tanaka, and H.H. Dearman, *J. Inorg. Nucl. Chem.*, **37**, 1511 (1975).
4) F.H. Bernhardt, N. Erdin, H. Staudinger, and V. Ullrich, *Eur. J. Biochem.*, **35**, 126 (1973).
5) M. Kunishima, Y. Sugiura, M. Takimoto, and H. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **24**, 1343 (1976).
6) Y. Sugiura, K. Ishizu, T. Kimura, and H. Tanaka, *Bioinorg. Chem.*, **4**, 291 (1975).

The incubation system contained 0.2 mM of the metal complex and 2.0 mM of acetanilide labelled with ^{14}C-acetanilide in 50% (v/v) acetone–water solution at pH 9.0. The reaction solution containing sulfide-incorporated complex was incubated for 90 min, and the solution containing selenide-incorporated complex was incubated for 60 min at 20°. After the incubation, the reaction products were separated and identified by thin-layer chromatography, using a solvent system of benzene: methanol: acetic acid=45: 8: 4. The yields of *o*-acetaminophenol and *p*-acetaminophenol formed were determined by measurement of ^{14}C-radioactivity with a Beckman liquid-scintillation counter, model LS-233 and an Aloka 2π thin-layer radiochromatoscanner. All values represent averages of several experiments.

Results and Discussion

As shown in Fig. 1, acetanilide was hydroxylated at an aromatic position to form *o*- and *p*-acetaminophenols. When DTT-Fe-S complex was used as the catalyst, *p*-aminophenol was also produced with trace amounts. On the other hand, ferric chloride alone showed no catalytic activity for hydroxylation of acetanilide under the same condition. No formation of *m*-acetaminophenol was found in the present catalyst systems, though *m*-acetaminophenol has been detected as one of hydroxylation products in liver microsomes of benzpyrene-induced rats.[7] Daly, *et al.* reported that acetanilide was metabolized to form *p*-acetaminophenol, *o*-acetaminophenol, and trace amount of aniline in rat liver microsomes.[8] In contrast, our catalytic system resulted in no formation of aniline during the acetanilide hydroxylation reaction.

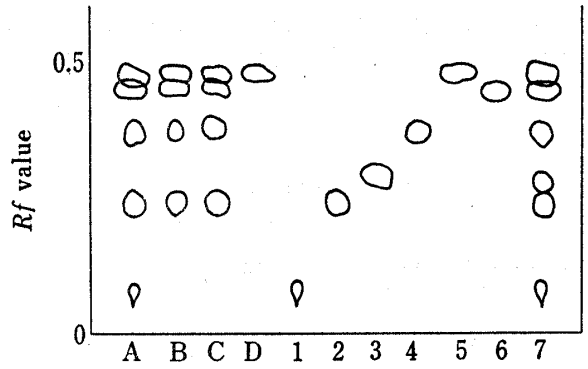

Fig. 1. Thin-Layer Chromatogram Obtained from Incubation of Acetanilide and Iron Complexes

The A–D represent the chromatogram of the following systems: A, acetanilide and DTT–Fe–S complex; B, acetanilide and DTT–Fe complex; C, acetanilide and DTT–Fe–Se complex; D, acetanilide and Fe. Reference compounds were 1, *p*-aminophenol; 2, *p*-acetaminophenol; 3, *m*-acetaminophenol; 4, *o*-acetaminophenol; 5, acetanilide; 6, dithiothreitol; 7, mixture of compounds 1—6. The chromatogram was obtained by use of benzene: methanol: acetic acid=45: 8: 4 for one hour.

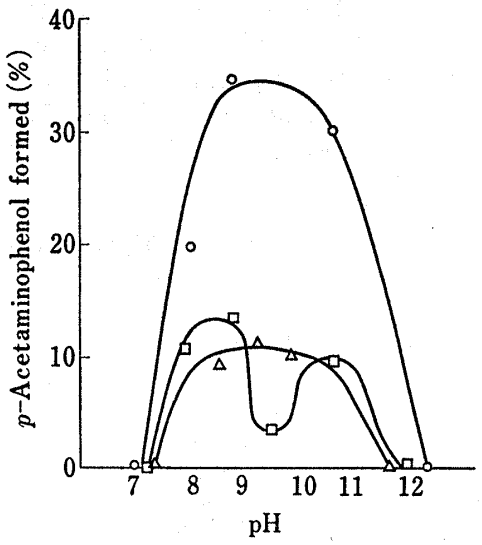

Fig. 2. Effect of pH on Acetanilide Hydroxylation Reaction by DTT-Fe Complexes

The iron complex used were as follows: DTT–Fe–S (○), DTT–Fe (□), and DTT–Fe–Se (△).

Figure 2 shows the pH-dependence of acetanilide hydroxylation by various iron complexes of DTT. The maximal hydroxylation activities by DTT-Fe-S and DTT-Fe-Se complexes were obtained in pH regions from 8.5 to 10.5 (DTT-Fe-S) and from 8.0 to 10.5 (DTT-Fe-Se) respectively, and that by DTT-Fe complex was observed in pH regions from 8.0 to 8.8 and from 10.0 to 11.2 separately. On the other hand, the optimum pH region for the complex formation of these iron complexes was approximately between 8.0 and 11.0. Thus pH effects both on

7) V. Ullrich, J. Wolf, E. Amadori, and H. Standinger, *Hoppe-Seyler Z. Physiol. Chem.*, **349**, 85 (1968).
8) J. Daly, D. Jerina, and B. Witkop, *Arch. Biochem. Biophys.*, **128**, 517 (1968).

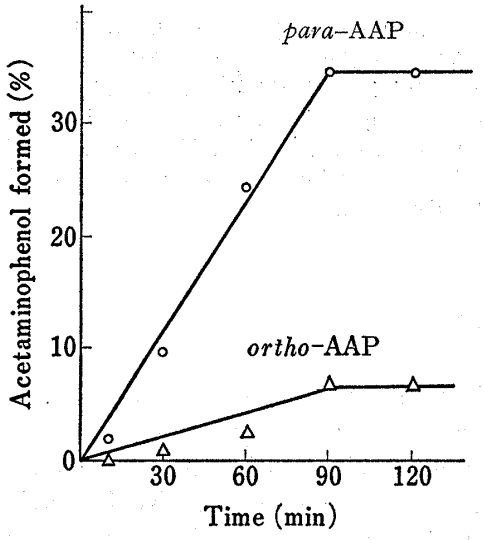

Fig. 3. Time-Cource of Acetaminophenol Formed During Acetanilide Hydroxylation Reaction by DTT-Fe-S Complex

acetanilide hydroxylation reaction and iron complex formation were substantially parallel. We have reported that the pH effect on O_2-uptake of these iron complexes is also dependent upon the complex formation.[6] These facts indicate that the iron complex and dissolved oxygen play an important role for the acetanilide hydroxylation reaction.

Figure 3 represents the time-course of the acetanilide hydroxylation reaction by DTT-Fe-S complex. The red solution of DTT-Fe-S complex decomposes to colorless solution above 90 min. After the complex decomposed, the hydroxylation reaction no longer proceeded.

As well as DTT-iron complexes, GSH-iron complexes also catalyzed the acetanilide hydroxylation reaction. Table I summarizes the results of the acetanilide hydroxylation catalyzed by these iron complexes. DTT-Fe-S and GSH-Fe-S complexes showed the highest activity among these iron complexes. As regard to DTT-iron complexes, the acetanilide hydroxylation was more effectively catalyzed by DTT-Fe-Se complex than by DTT-Fe complex, whereas it was in reverse in GSH-iron complex systems. This phenomenon may be explained by the fact that GSH-Fe complex solution containing excess GSH releases inorganic sulfur, incorporates the released sulfur, and that is spontaneously converted into GSH-Fe-S complex.[3a] The percentage of *o*-acetaminophenol formed was approximately 5%, regardless of variety of the complex used.

Table I. Hydroxylation of Acetanilide by Various Iron Complexes

Complex	*p*-Acetaminophenol formed (%)	*o*-Acetaminophenol formed (%)	*para*/*ortho* ratio
DTT-Fe	8.2	4.7	1.74
DTT-Fe-S	34.8	6.9	5.04
DTT-Fe-Se	11.1	5.0	2.22
GSH-Fe	21.8	6.3	3.46
GSH-Fe-S	31.4	5.1	6.16
GSH-Fe-Se	15.5	5.6	2.77

Table II. Rates of Acetanilide-Hydroxylation by Various Iron Complexes

Complex	Turnover number	
	Mol *p*-acetaminophenol/min/mol chelate	Mol *o*-acetaminophenol/min/mol chelate
DTT-Fe	9.1×10^{-3}	5.2×10^{-3}
DTT-Fe-S	3.9×10^{-2}	7.7×10^{-3}
DTT-Fe-Se	1.9×10^{-2}	8.3×10^{-3}
GSH-Fe	2.4×10^{-2}	7.0×10^{-3}
GSH-Fe-S	3.5×10^{-2}	5.7×10^{-3}
GSH-Fe-Se	2.6×10^{-2}	9.3×10^{-3}

Table II shows the rates of the hydroxylation of acetanilide by various iron complexes. It has been reported by Lu, *et al.*[9] and Mieyal, *et al.*[10] that rates of aniline hydroxylation catalyzed by the reconstituted cytochrome P-450 systems are 0.22—0.37 (mol *p*-aminophenol/min/mol hemoprotein) and those by the reconstituted cytochrome P-450 systems containing

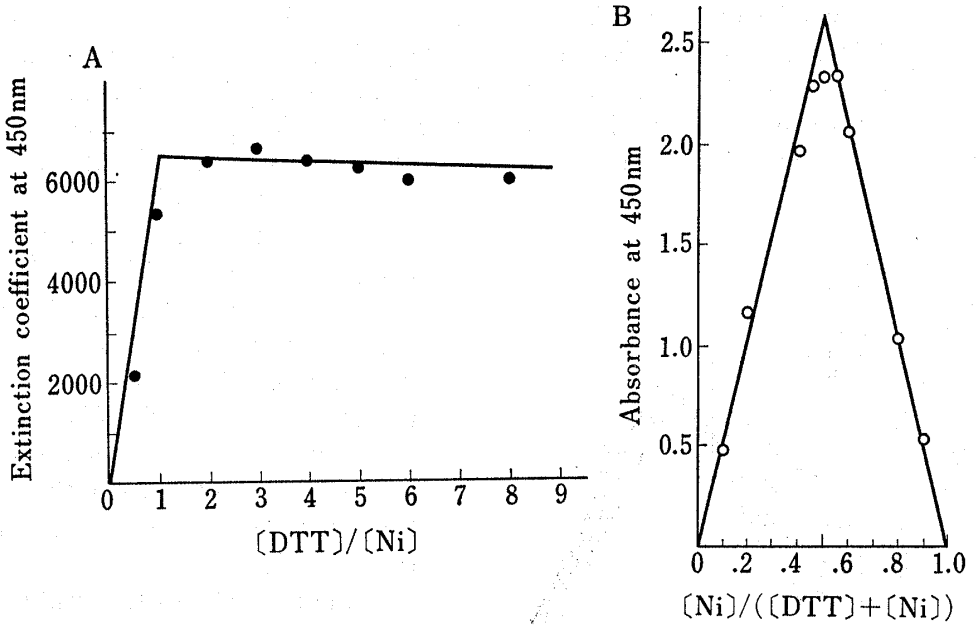

Fig. 4. Molar Ratio (A) and Continuous Variation (B) Methods between DTT and Nickel

The concentration of the reagents were as follows: (A), [Ni]=2×10^{-4} M and (B), [DTT]+[Ni]=10^{-3} M.

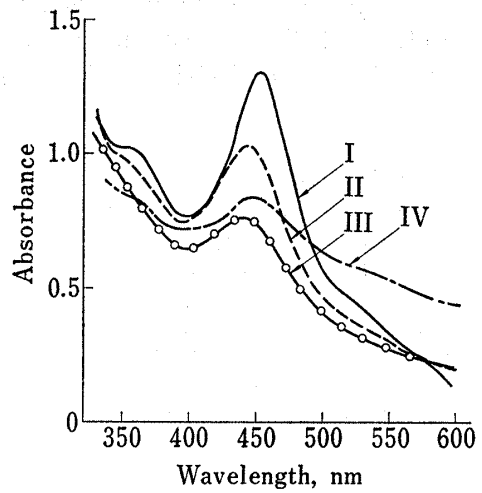

Fig. 5. Visible Absorption Spectra of DTT-Ni Complexes at pH 9.2

(I), DTT-Ni complex; (II), DTT-Ni-S complex; (III), DTT-Ni-S (excess) complex; (IV), DTT-Ni-Se complex. The spectra were measured by mixing following materials: (I), DTT(2.0 mM) and $NiCl_2$(0.2 mM); (II), DTT (2.0 mM), Na_2S (0.2 mM), and $NiCl_2$ (0.2 mM); (III), DTT (2.0 mM), Na_2S (2.0 mM), and $NiCl_2$(0.2 mM); (IV), DTT(2.0 mM), H_2SeO_3(0.2 mM), and $NiCl_2$(0.2 mM).

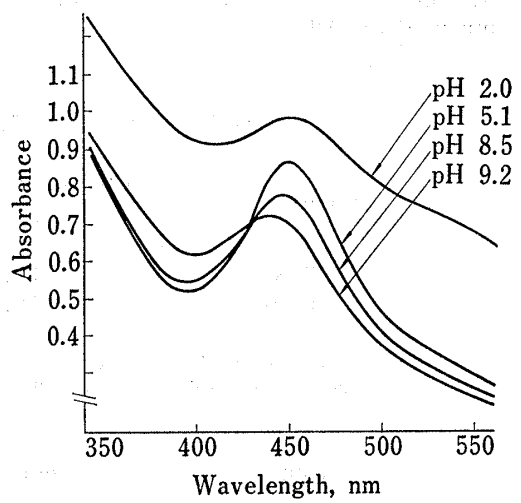

Fig. 6. Visible Absorption Spectra of DTT–Ni–S Complex at Various pH

The sample solution contained DTT (2.0 mM), Na_2S (2.0 mM), and $NiCl_2$ (0.2 mM).

9) A.Y.H. Lu, M. Jacobson, W. Levin, S.B. West, and R. Kuntzman, *Arch. Biochem. Biophys.*, **153**, 294 (1972).
10) J.J. Mieyal, R.S. Ackerman, J.L. Blumer, and L.S. Freeman, *J. Biol. Chem.*, **251**, 3436 (1976).

hemoglobin are 0.08—0.23. These turnover numbers are appreciably close to those obtained in this work. It is well-known that acetanilide is mainly hydroxylated at the *p*-position by liver microsomal preparations in the presence of NADPH and molecular oxygen.[11] In our model systems, however, the acetanilide hydroxylation was proceeded without NADPH. Addition of NADPH to these reaction systems gave no effect on the yield of this hydroxylation reaction. Considering that NADPH participates in the reaction as an electron donor in biological systems, excess thiol ligand may participate as an electron donor in the present systems. These hydroxylation reactions seem to be mainly electrophilic, since acetanilide is hydroxylated at *para* and *ortho* positions and no *meta* position is hydroxylated.

Figure 4 clearly shows the existence of 1:1 complex between nickel and DTT.

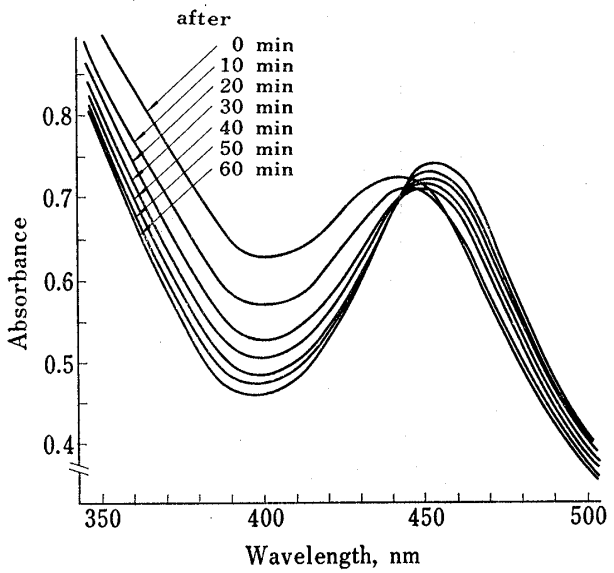

Fig. 7. Sprectral Change with Time of DTT–Ni–S Complex at pH 9.2

When inorganic sulfur was added to DTT-Ni complex, the spectrum of this complex was changed with disappearance of shoulder at 350 nm and blue shift of absorption maximum at 450 nm. The fact reveals that inorganic sulfur was incorporated into DTT-Ni complex. Addition of inorganic selenide (H_2SeO_3) to DTT-Ni complex caused the red shift of the absorption from 450 nm to 458 nm (see Fig. 5).

Figure 6 shows the visible spectra of DTT-Ni-S complex at various pH. Yellow-orange DTT-Ni-S complex exhibits the spectrum with absorption maximum at 440 nm at pH 9.

When pH of the solution is acidic, the spectrum of DTT-Ni-S complex bacame similar to that of DTT-Ni complex. The

Table III. Hydroxylation of Acetanilide by Nickel Complexes

Complex	*p*-Acetaminophenol formed (%)	*o*-Acetaminophenol formed (%)	*para*/*ortho* ratio
DTT–Ni	30.0	7.2	4.17
DTT–Ni–S	22.3	6.0	3.72

The reaction solutions contained ^{14}C-acetanilide(2.0 mM) and Ni complexes (0.2 mM) at pH 9.0, and were incubated for 120 min at 20.

Table IV. Rates of Acetanilide–Hydroxylation by Nickel Complexes

Complex	Turnover number	
	Mol *p*-acetaminophenol/ min/mol chelate	Mol *o*-acetaminophenol/ min/mol chelate
DTT–Ni	2.5×10^{-2}	6.0×10^{-3}
DTT–Ni–S	1.9×10^{-2}	5.0×10^{-3}

11) *a*) H.S. Posner, S. Mitoma, and S. Udenfriend, *Arch. Biochem. Biophys.*, **94**, 269 (1961); *b*) K. Krisch and H. Staudinger, *Biochem. Z.*, **334**, 312 (1961).

same phenomenon was observed, when DTT-Ni-S complex was permitted to stand for one hour (see Fig. 7).

Of special interest is the high catalytic activity of DTT-Ni complexes as well as the iron complexes for the hydroxylation of acetanilide. Tables III and IV show the experimental results. Nickel ion alone gave no catalytic effect. On the other hand, it has been reported that acetanilide is hydroxylated by various metal ions such as Cu^{+}, Ti^{3+}, V^{3+}, and Sn^{2+}, and that *o*-, *m*-, and *p*-acetaminophenols are formed.[12] Hydroxylation and *o*-dealkylation of phenacetin are also proceeded by reduced metal complex (Fe^{2+}, Cu^{+}, Ti^{3+}, V^{2+}, Sn^{2+}) plus O_2 system.[13] The DTT-Ni complex plus O_2 system may participate in the present hydroxylation. In fact, several oxygen complexes of nickel have been prepared and characterized.[14] However, further investigations are necessary to clarify the reaction mechanism for the acetanilide hydroxylation by DTT-Ni complex systems.

12) H. Staudinger and V. Ullrich, *Z. Naturforschg.*, **19B**, 409 (1964).
13) V. Ullrich, D. Hey, H. Staudinger, H. Buch, and W. Rummel, *Biochem. Pharmacol.*, **16**, 2237 (1967).
14) *a*) S. Otsuka, A. Nakamura, and Y. Tatsuno, *J. Am. Chem. Soc.*, **91**, 6994 (1969); *b*) G. Wilke, H. Scott, and P. Heimbach, *Angew. Chem. (Int. Ed. Eng.)*, **6**, 92 (1967).