[Chem. Pharm. Bull. 26(6)1713—1717(1978)]

UDC 547.546.03.04 : 577.158.08

Susceptibility of Aromatic Nitro Compounds to Xanthine Oxidase-catalyzed Reduction[1]

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(Received October 29, 1977)

The reduction of aromatic nitro compounds was investigated in purified milk xanthine oxidase-xanthine system. The *p*-substituted nitro compounds with electron-withdrawing groups served as an efficient acceptor of electron, whereas those with electron-repelling groups were less active. The reduction products formed from some nitro compounds were also examined by thin-layer chromatography and spectrophotometry. As a result, the corresponding hydroxylamines were identified in all cases. Furthermore, the reduction of *ortho*, *meta* and *para* isomers of some nitro compounds was investigated in the same manner. The reduction rate was greatest at *para* substitution of electron-withdrawing group and at *meta* substitution of electron-repelling group, respectively.

Keywords——aromatic nitro compound; milk xanthine oxidase; nitro-reduction; electron-withdrawing substituent; electron-repelling substituent; hydroxylamino compound; reduction product

The aromatic nitro compounds form a group of substances of considerable practical importance to man and their industrial production often entails potential health hazards for persons employed in their manufacture. The reduction of nitro groups of these compounds is a well-known metabolic reaction which appears to be essential for the occurrence of their biological activity. Several enzymes have been shown to possess significant nitro-reductase activity for a various kind of nitro compounds *in vitro*. They are NADPH-cytochrome c reductase,[3–5] cytochrome P-450,[6] DT-diaphorase,[7,8] lipoyl dehydrogenase,[9] aldehyde oxidase[10] and xanthine oxidase.[5,9,11–15] Among these enzymes, xanthine oxidase (EC 1.2.3.2) which is a molibdoflavoprotein present in various tissues of animals is known to catalyze the reduction of a wide variety of substances including NAD, artificial dyes, quinones, ferricyanide, cytochrome c, nicotinamide N-oxide, purine N-oxide[16] and heterocyclic compounds such as nitrofuran derivatives[5,11,14,15] and niridazole.[13] In addition, the enzyme catalyzes the reduction of a few aromatic nitro compounds, 2,4-dinitrophenol[12] and 2,4,6-trinitrotoluene,[9] but

1) This work was presented at 8th Symposium on Drug Metabolism and Action, Hiroshima, November, 1976.
2) Location: *Maidashi, Higashi-ku, Fukuoka, 812, Japan.*
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15) K. Tatsumi, S. Kitamura, and H. Yoshimura, *Arch. Biochem. Biophys.*, **175**, 131 (1976).
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not the reduction of *p*-nitrophenol,[13] *p*-nitrobenzoate[13,17] and chloramphenicol.[13,17] The relationship between the enzymatic reduction and the structure of aromatic nitro compounds remains unknown. The present paper records studies on the reduction of various aromatic nitro compounds in purified milk xanthine oxidase-xanthine system. It will be shown that a degree of reduction is influenced strongly by the electronic property and the position of substituent in benzene ring.

Materials and Methods

Chemicals——*p*-Nitro-*p'*-hydroxyphenyl ether (mp 171—172°) was synthesized by the method of Igawa[18] and *p*-hydroxylaminonitrobenzene (mp 104—107°) by the method of Kuhn and Weygand.[19] *p*-Hydroxylaminoacetophenone (A) and methyl *p*-hydroxylaminobenzoate (B), new hydroxylamino compounds, were synthesized according to a slight modification of the method of Kamm[20] as follows: A mixture of *p*-nitroacetophenone (6.5 g) or methyl *p*-nitrobenzoate (7.2 g), ammonium chloride (3.4 g) and water (100 ml) was stirred vigorously by means of a mechanical stirrer, and zinc dust (8.0 g) was added during the course of 15 min. Stirring was continued for 15 min after all the zinc dust had been added. The solution was filtered in order to remove the zinc oxide, which was washed with hot water. The filtrate was saturated with sodium chloride (75 g) and cooled to 0°. Each of the hydroxylamines which crystallized out was filtered and recrystallized from benzene. (A)[21]: mp 110—116°. *Anal.* Calcd. for $C_8H_9NO_2$: C, 63.56; H, 6.00; N, 9.27. Found: C, 63.81; H, 6.10; N, 9.25. IR ν_{max}^{Nujol} cm^{-1}: 3240, 1640, 1190. NMR δ: 2.24 (3H, singlet, CH_3), 7.32 (4H, quartet, aryl H), 8.76 (1H, broad singlet). MS *m/e*: 151 (M^+). (B): mp 109—112°. *Anal.* Calcd. for $C_8H_9NO_3$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.25; H, 5.62; N, 8.43. IR λ_{max}^{KBr} cm^{-1}: 3340. 3250, 1680, 1175. NMR δ: 3.26 (3H, singlet, CH_3), 7.28 (4H, quartet, aryl H). MS *m/e*: 167 (M^+). *p*-Nitrobenzenesulfonamide, *p*-nitrosobenzenesulfonamide, *p*-hydroxylaminobenzenesulfonamide and *p*-azoxybenzenesulfonamide were kindly donated by Dr. I. Yamamoto of Faculty of Pharmaceutical Sciences, Hokuriku University. The aromatic nitro compounds shown in Table II, except *p*-nitro-*p'*-hydroxylphenyl ether and *p*-nitrobenzenesulfonamide, were purchased from Tokyo Chemical Industry Co., Ltd. *p*-Aminoacetophenone, methyl *p*-aminobenzoate and *p*-aminobenzenesulfonamide were obtained from Wako Pure Chemical Industries, Ltd. All chemicals were reagent grade. Purified milk xanthine oxidase (No. X-1875) was purchased from Sigma Chemical Co., Ltd.

Assay of Reducing Activity——A Thunberg-type cuvette was used in this experiment. The reaction mixture consisted of 10 μl of a methanolic solution of aromatic nitro compound (0.075 μmol), 30 μl of a 0.1 N NaOH solution of xanthine (0.15 μmol), 60 μl of milk xanthine oxidase (0.05 units) and 1/30 M phosphate buffer (pH 7.4) to a final volume of 2.5 ml. Prior to incubation, the cuvette was gassed for 3 min with nitrogen and then evacuated with a water aspirator for 3 min. After the cuvette was preheated for 5 min at 37°, the reaction was started by addition of the enzyme solution from the side arm to the cuvette. The relative reduction rate of nitro compound, expressed as nanomoles of uric acid formed from xanthine per milligram of protein per minute, was calculated from the initial linear phase of the curve which was obtained by measuring the increase in absorbance at 292 nm (the absorption maximum of uric acid) with a Hitachi 124 spectrophotometer. In the absence of an electron acceptor, no uric acid was formed under such conditions. The assay method of the hydroxylamino or the nitroso compound reductase activity is essentially identical to that described above for the nitro compound reductase.

Identification of Reduction Products——The reduction products were identified by TLC and UV spectrophotometry as follows: The reaction mixture, after incubation for 30 min, was extracted twice with an equal volume of ethyl acetate. The combined extracts were concentrated to a small volume under vacuum and then chromatographed on thin-layer plates of silica gel (Wako gel B-5FM, 0.25 mm thick). The solvent systems and visualization methods used were shown in Table I in which *Rf* values of authentic samples of some aromatic nitro compounds and their expected reduction products are summarized.

Furthermore, in order to examine spectrophotometrically, the spot of reduction product which was detected under UV light was scrapped and eluted with ethanol. The UV absorption maxima of authentic samples of some hydroxylamino compounds in ethanol are as follows: *p*-Hydroxylaminobenzenesulfonamide

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20) O. Kamm, "Organic Synthesis," 2nd ed., Coll. Vol. I, ed. by H. Gilman, John Wiley and Sons, Inc., New York, 1956, pp. 445—447.
21) The spectra were recorded on the following instruments: IR, Japan Spectroscopic DS-701 G IR spectrophotometer; mass, Japan Electron Optics (JEOL) JMS-01SG mass spectrometer; NMR, Japan Electron Optics (JEOL) JNM PS-100 spectrometer using tetramethylsilane as an internal standard and DMSO-d_6 as a solvent.

Table I. *Rf* Values of Aromatic Nitro Compounds and Their Expected Reduction Products in Thin-Layer Chromatography

Compound	*Rf*	Solvent	Visualized with
p-Nitroacetophenone	0.60	A	III
p-Hydroxylaminoacetophenone	0.27	A	I, II, III
p-Aminoacetophenone	0.42	A	I, III
Methyl *p*-nitrobenzoate	0.58	B	III
Methyl *p*-hydroxylaminobenzoate	0.23	B	I, II, III
Methyl *p*-aminobenzoate	0.32	B	I, III
p-Dinitrobenzene	0.75	A	III
p-Hydroxylaminonitrobenzene	0.35	A	I, II, III
p-Nitroaniline	0.46	A	I, II
p-Nitrobenzenesulfonamide	0.60	C	III
p-Nitrosobenzenesulfonamide	0.55	C	I, II, III
p-Hydroxylaminobenzenesulfonamide	0.31	C	I, II, III
p-Aminobenzenesulfonamide	0.40	C	I, III
p-Azoxybenzenesulfonamide	0.45	C	III

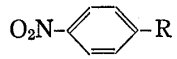
Solvent: (A) $CHCl_3$-MeOH (9:1), (B) benzene-acetone (9:1), (C) $CHCl_3$-MeOH (4:1).
Visualization: (I) Ehrlich reagent (5% methanolic solution of *p*-dimethylaminobenzaldehyde-conc. HCl, 3:1), (II) 1% aqueous solution of sodium pentacyanoammineferroate, (III) UV light (3650 Å).

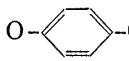
225 nm, *p*-hydroxylaminoacetophenone 311 nm, methyl *p*-hydroxylaminobenzoate 288 nm and *p*-hydroxylaminonitrobenzene 368 nm.

Results

As shown in Table II, a remarkable difference of the reduction rate was found with *p*-substituted aromatic nitro compounds.

Table II. Influence of *p*-Substituents on Reduction of Aromatic Nitro Compounds by Xanthine Oxidase



R	Reduction rate[a]	σ_p[b]	R	Reduction rate[a]	σ_p[b]
	12	—	COO^-	80	0.12
			Cl	159	0.23
NH_2	23	−0.17	Br	171	0.23
OCH_3	26	−0.27	SO_2NH_2	177	0.62
OH	29	−0.37	CN	476	0.66
SH	29	0.15	$CONH_2$	513	0.36
CH_3	29	−0.17	$COOCH_3$	527	0.39
SO_3^-	38	0.09	NO_2	633	0.78
H	48	—	$COCH_3$	656	0.50
	60	−0.01	CHO	684	0.22
CH_2Cl	63	0.18		713	0.36
CH_2OH	77	0.08			

Each value represents mean of three experiments.
a) The reduction rate is expressed as nanomoles of uric acid formed from xanthine per milligram of protein per minute.
b) Values of σ_p are quoted from Lange's Handbook of Chemistry, Vol. 3, ed. by J.A. Dean, McGraw-Hill, New York, 1973, pp. 3-128—3-131.

The nitro compounds with electron-withdrawing substituents (CN, NO_2, $COOCH_3$ *etc.*) served as an efficient acceptor of electron, whereas those with electron-repelling substituents (NH_2, OH, CH_3 *etc.*) were less active. The σ value of the Hammett equation which represents

the characteristic substituent constant is convenient for evaluation of electronic property of substituent; the value becomes higher in more strongly electron-withdrawing substituent. Table II also shows that there is some correlation between the reduction rates and the σ values of *p*-substituents. From these results, it was concluded that the reduction of *p*-substituted aromatic nitro compounds by xanthine oxidase are influenced strongly by the electronic property of substituents.

Although all of these compounds were not examined, the reduction products formed from *p*-nitroacetophenone, methyl *p*-nitrobenzoate, *p*-dinitrobenzene and *p*-nitrobenzenesulfonamide during a 30 min incubation under such conditions were studied by both TLC and spectrophotometry as described in Methods. As a result, the *Rf* values and UV spectra of the reduction products of these nitro compounds were identical in all respects with those of the authentic samples of the corresponding hydroxylamino compounds. In the case of *p*-nitrobenzenesulfonamide reduction, a small amount of *p*-azoxybenzenesulfonamide was also detected by TLC. Thus, aromatic nitro compounds were ultimately reduced to the hydroxylamines, but not to the amines, under these conditions. The results were supported by the finding that these hydroxylamino compounds were resistant to further reduction under the identical conditions. The reduction rates, expressed as nanomoles of uric acid formed from xanthine per milligram of protein per minute, were as follows: *p*-Hydroxylaminoacetophenone 14, methyl *p*-hydroxylaminobenzoate 20, *p*-hydroxylaminoaniline 17 and *p*-hydroxylaminobenzenesulfonamide 6.

The reduction of the nitro group to the hydroxylamino group might be expected to take place in two stages, involving the intermediate formation of the nitroso group. We estimated that the reduction of *p*-nitrosobenzenesulfonamide to the hydroxylamino compound occurred very rapidly than the reduction of the *p*-nitrobenzenesulfonamide.[22] The conversion of aromatic nitro to nitroso compound, therefore, appears to be the rate-limiting step in the reduction sequence by xanthine oxidase. This evidence is in accord with the view that the reduction of the nitro to the nitroso group seems to be a rate-limiting step in nitrobenzene reduction in microsomal reduction system, which was reported by Gillette, *et al.*[6]

We further investigated the reduction of *ortho*, *meta* and *para* isomers of some nitro compounds by xanthine oxidase. As shown in Table III, all three isomers of each nitro compound were reduced at varying degrees. Reduction rate of nitrochlorobenzene or nitrobenzaldehyde was highest with the *para* isomer; the *ortho* compound was less readily reduced by the enzyme than its *para* and *meta* isomers, indicating the steric hindrance effect at *ortho* position. The *para* compound of dinitrobenzene was also more readily reduced than its isomers. In this case, the effect of *ortho* steric hindrance on reduction was not so remarkable as the *ortho* compounds described above. Although all isomers of nitroaniline were consider-

Table III. Influence of Position of Substituents on Reduction of Aromatic Nitro Compounds by Xanthine Oxidase

	O_2N-C$_6$H$_4$-Cl	O_2N-C$_6$H$_4$-CHO	O_2N-C$_6$H$_4$-NO_2	O_2N-C$_6$H$_4$-NH_2
	Reduction rate[a]			
Para	159	684	633	23
Meta	69	143	197	63
Ortho	44	83	342	7

Each value represents mean of three experiments.
a) The reduction rate is expressed as nanomoles of uric acid formed from xanthine per milligram of protein per minute.

22) When *p*-nitrosobenzenesulfonamide was incubated with xanthine oxidase under the conditions described in Methods, it was reduced to the hydroxylamine too fast to be capable of determining the reduction rate.

ably resistant to reduction, the *meta* compound was more readily reduced than its *para* and *ortho* isomers. These facts indicate that the reduction of the isomers of aromatic nitro compounds by xanthine oxidase was greatest at *para* substitution of electron-withdrawing group and at *meta* substitution of electron-repelling group, respectively.

Discussion

Previous works showed that with xanthine oxidase system, only a limited range of aromatic nitro compounds, namely 2,4-dinitrophenol[12] and 2,4,6-trinitrotoluene,[9] could act as an electron acceptor, but *p*-nitrophenol,[13] *p*-nitrobenzene[13,17] and chloramphenicol[13,17] did not appear to be reduced. In the present study, the structural requirements for such activity was therefore examined. As a result, it was found that the reduction of aromatic nitro compounds was influenced greatly by the electronic property and the position of substituents, and the compounds with strongly electron-withdrawing substituents in *para* position of benzene ring were reduced by xanthine oxidase approximately to the same extent as nitrofuran derivatives[23] which are well known to undergo reduction in this enzyme system.

Like xanthine oxidase, aldehyde oxidase (EC 1.2.3.1) is a molybdenum-containing flavoprotein known to be present in mammalian cells and can reduce nitrofurazone, but not *p*-nitrobenzoate and *p*-nitroanisole.[10] Considering from such similarity of both enzymes, aldehyde oxidase-catalyzed reduction of aromatic nitro compounds appears to proceed similarly as that with xanthine oxidase.

In addition, the present study provided the evidence for the identification of hydroxylamine analogues as a major reduction product of some nitro compounds. Previously, Bueding and Jolliffe[9] also demonstrated that 2,4,6-trinitrotoluene was reduced to 4-hydroxylamino-2,6-dinitrotoluene by xanthine oxidase, but no amine formation was observed with this enzyme. However, we can not conclude that aromatic nitro compounds are always reduced to the corresponding hydroxylamines by xanthine oxidase system, for only few nitro compounds have been examined for their reduction products.

In the case of *p*-nitrobenzenesulfonamide, a small amount of azoxy-compound was detected by TLC as one of reduction products. So far it is known that aromatic hydroxylamines are extremely labile and some derivatives are very readily converted into azoxy-compounds.[24] Although no drastic procedure during the isolation was used in the present experiments, it is most probable that the azoxy-compound was formed during the isolation procedure and was not originally formed by the action of xanthine oxidase. In fact, the formation of such azoxy-compound was reported to occur during the isolation of the reduction product of 2,4,6-trinitrotoluene.[9]

Acknowledgement We thank Miss M. Yasuda for her excellent technical assistance. We also wish to thank Dr. I. Yamamoto for the generous supplies of *p*-nitrobenzenesulfonamide and its related compounds. This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture which is acknowledged.

23) When assayed under the same conditions as described in Text, the reduction rates of some antibacterial nitrofuran derivatives, expressed as nanomoles of uric acid formed from xanthine per milligram of protein per minute, are as follows: Nitrofurazone 456, nitrofurantoin 633, furazolidone 471 and AF-2 (2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide) 314.

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