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56. Morizo Ishidate*[1] and Akira Hanaki*[2] : Oxidative N-Demethylation of Aminoazo Compounds by Model System. I. Oxidation of 4-N-Dimethylaminoazobenzene by Some Systems.

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The potent hepatocarcinogen, 4-N-dimethylaminoazobenzene (DAB), is subject to at least three metabolic reactions in the intact rat[1]; oxidative N-demethylation of the dimethylamino group, hydroxylation at 4′ and/or 3′ position, and reductive cleavage of the azo bond. Among these metabolic reactions, which have been demonstrated also in fortified rat liver homogenates,[2] N-demethylation is the most interesting with respect to carcinogenesis, because this reaction has been assumed to be concerned most intimately with the formation of protein-bound aminoazo dyes[3,4] observed in the rat liver, which is the only organ susceptible to the carcinogen. However, the above metabolic reactions are all carried out in the same cell component (microsomes) and require a common cofactor reduced type of triphosphopyridine nucleotide (TPNH). With the preparation of liver microsomes, therefore, it is difficult to discuss the N-demethylation of DAB without considering the accompanying metabolism. Accordingly, investigation of the chemical oxidation which might resemble the biological oxidation becomes necessary.

Several model systems which catalysed the hydroxylation of aromatic amines were reported by Udenfriend[5] and Boyland,[6] while only one N-demethylating system using the Milas reagent was reported by Anderson.[7] The present work was undertaken in the hope that information about the N-demethylation of DAB and its derivatives using a suitable model system might shed some light on the bound-dye formation, and thereby lead to some clues on the mechanism of carcinogenesis. Because of the difficulty of getting the complete system, it seemed desirable first to take simpler models and discuss them in comparison with the N-demethylase system located in liver microsomes.

Table I. Oxidation of MAB with Hydrogen Peroxide

Oxidation system	Yield of oxidation product (%)	
	AB	4′-OH-MAB
H_2O_2	0.4	—
H_2O_2-ascorbic acid	0.9	0.1
Fe^{2+}-H_2O_2	1.3	0.1
Fe^{2+}-H_2O_2-ascorbic acid	2.4	0.2

The systems contain 8.0×10^{-5} moles MAB, 8.0×10^{-5} moles Fe^{2+} as sulfate, 1.5×10^{-4} moles EDTA,[a] 4.0×10^{-4} moles ascorbic acid, 1.6×10^{-3} moles H_2O_2 in total volume of 30 cc. (30% *tert*-BuOH). The mixture was shaken for 2 hr. at 40° and pH 6.8 (0.05*M* phosphate buffer as finite concentration).

a) Ethylenediaminetetraacetic acid

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Table II. Oxidation of Aminoazo Dyes with Alkaline Peroxydisulfate

Substrate	Hydroxylation (%)	Demethylation (%)
DAB	15.5	1.9
MAB	21.2	3.0
AB	3.8	

The system contains 1.0×10^{-4} moles aminoazo dye, 1.2×10^{-4} moles potassium peroxydisulfate, and 20 cc. of phosphate buffer (pH 11.2) ina total volume of 50 cc. The mixture was shaken for 4 hr. at 40°.

Table III. Autoxidation of DAB

Oxidation system	Yield of oxidation product (%)	
	MAB	4'-OH-DAB
Fe^{2+}-O_2	0.2	—
Fe^{2+}-O_2-ascoric acid	2.4	0.3

The systems contain 5.0×10^{-5} moles DAB, 1.5×10^{-4} moles Fe^{2+} as sulfate, 6.0×10^{-4} moles EDTA, and 3.0×10^{-3} moles ascorbic acid in total volume of 250 cc. (20% *tert*-BuOH). The mixture was shaken at 40° and pH 6.8 (0.05*M* phosphate buffer as finite concentration).

Experimental

Oxidation of Aminoazo Compounds——The oxidation system given in Tables I, II, or III was incubated in a metabolic shaker, designed for this purpose, at 40°. The reaction was initiated by adding the oxidizing agent and stopped by adding cracked ice. After the reaction, dyes including both substrate and products were extracted several times with $CHCl_3$.

Separation and Determination of Oxidation Products——The oxidation products including demethylated aminoazo dyes, hydroxylated aminoazo dyes, and 5-phenylazo-2-aminophenyl sulfates (sulfateester) were separated and determined by the following procedures.

hydroxylated derivatives of aminoazo dyes : The above dye-containing $CHCl_3$ solution was extracted with 1*N* NaOH. The aqueous layer was separated, slightly acidified with 10% HCl, excess of 0.5% $NaHCO_3$ solution was added, and reextracted several times with $CHCl_3$. The combined $CHCl_3$ extract was evaporated to dryness in à reduced pressure and the residue was dissolved in 2*N* HCl-5% EtOH. The amount of the dye produced was determined by measuring the optical absorbancy at the specific wave length; λ_{max} : 4'-OH-DAB : 550 mμ ($E^{\gamma/cc.}_{1 cm}$ 0.0781); 4'-OH-MAB : 550 mμ ($E^{\gamma/cc.}_{1 cm}$ 0.118).

Demethylated aminoazo dyes : An aliquot of the $CHCl_3$ extract was evaporated to dryness in a reduced pressure, the residue was dissolved in light petroleum (b.p, 60~80°), and subjected to chromatography using alumina column in order to separate the demethylated dye from the substrate. The light petroleum solution was poured on the alumina column and the column was developed with benzene-light petroleum mixture. MAB and AB were eluted with benzene-light petroleum (1:2 and 1:1, respectively) and the effluent was evaporated to dryness in a reduced presure. Determination of the demethylated dyes was carried out by the same procedure as described for hydroxylated aminoazo dyes. λ_{max} MAB : 510 mμ ($E^{\gamma/cc.}_{1 cm}$ 0.217); AB : 500 mμ ($E^{\gamma/cc}_{1 cm}$ 0.0935).

Sulfate esters : The reaction mixture was diluted with H_2O, extracted with $CHCl_3$ to remove the demethylated and hydroxylated dyes and the aqueous layer was extracted several times with Bu OH at pH 5~6. The combined BuOH extract was evaporated and the sulfate ester in the residue was determined by colorimetry as described above.

2-Dimethylamino-5-phenylazophenyl sulfate : $\lambda^{H_2O}_{max}$: 380 mμ(log ε 16,200), $\lambda^{1N \cdot HCl}_{max}$: 320 mμ (log ε 22,200);
2-Methylamino-5-phenylazophenyl sulfate : $\lambda^{H_2O}_{max}$: 400 mμ (log ε 22,800), $\lambda^{1N \cdot HCl}_{max}$: 513 mμ (log ε 51,300);
2-Amino-5-phenylazophenyl sulfate : $\lambda^{H_2O}_{max}$: 380 mμ (log ε 21,100), $\lambda^{1N \cdot HCl}_{max}$: 505 mμ (log ε 18,100).

Preparation of 4-N-Dimethylaminoazoxybenzene N-Oxide[8]——One equivalent of DAB was dissolved in benzene and 2 equivalent of perbenzoic acid in benzene solution was added with constant stirring. Instantly crystals appeared. After standing in a cold place for 2 hr., the crystals were collected, washed with benzene, and recrystallized from benzene, m.p. 135~137°.

8) P.H. Gore, O.H. Weeler : J. Am. Chem. Soc., 78, 2160 (1956).

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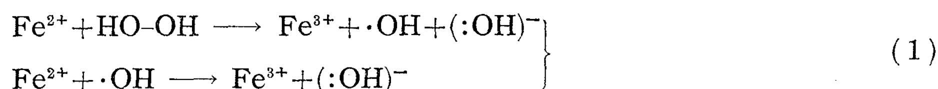
Result

(1) Oxidation with Hydrogen Peroxide

DAB was demethylated by hydrogen peroxide alone, yielding a very small amount of MAB, but the presence of a trace of metal ions appeared to accelerate this oxidation. Certainly, the Fenton's reagent, oxidation mechanism of which is given in Equation (1),[9] is shown to be more effective for the N-demethylation of DAB. In this case, iron (II) ion was assumed to catalyze the N-demethylation and the addition of an adequate reducing agent such as ascorbic acid, in order to maintain the ratio of iron (II) to iron (III) ion concentration high, favored the formation of MAB from DAB (Table I). Beside the N-demethylation, hydroxylation at 4′-position of DAB also took place in this system.



$$\left.\begin{array}{l} Fe^{2+} + HO\text{-}OH \longrightarrow Fe^{3+} + \cdot OH + (:OH)^- \\ Fe^{2+} + \cdot OH \longrightarrow Fe^{3+} + (:OH)^- \end{array}\right\} \quad (1)$$

(2) Oxidation with Peroxydisulfate

Peroxydisulfate in alkaline solution usually attacks the *ortho*-position with respect to the amino group of aromatic amines and forms 2-aminophenyl sulfates.[6] By this reagent, DAB was oxidized to its *o*-aminophenol analog, (2-dimethylamino-5-phenylazophenyl sulfate), and 2-methylamino-5-phenylazophenyl sulfate was obtained from MAB, which had been detected in the urinary sulfate esters from a dog fed with DAB.[10] Aminoazobenzene (AB), a very weak carcinogen, was less reactive against this reagent than the potent carcinogen such as DAB and MAB (Table II).

(3) Oxidation with Perbenzoic Acid

It is generally found that azo dyes and tertiary amines are oxidized with organic peracid to azoxy and amino-N-oxide compounds, respectively. With perbenzoic acid, DAB was quantitatively oxidized to 4-N-dimethylaminoazoxybenzene amino-N-oxide, which was not detected in urinary metabolites from animals fed with DAB.

(4) Autoxidation

Autoxidation of iron (II) to iron (III) ion is usually conjugated with the reduction of oxygen, which is assumed to be the activation of oxygen in the autoxidation system. When this activated oxygen attacks DAB, MAB is produced, but the rate of N-demethylation is too slow to be found quantitatively. In order to make the rate fast, it is probably useful to maintain the iron (II) ion concentration relatively high by reducing iron (III) to iron (II) ion. The modified system, which was fortified with ascorbic acid, was reported first as an aromatic hydroxylase model by Udenfriend and co-workers.[5] This fortified system did accelerate the oxidative N-demethylation of DAB as compared with the mere autoxidation system, though not so fast.

Discussion

The enzyme system which oxidatively N-demethylates DAB is located in the microsomes of a rat liver and requires both oxygen and TPNH.[11] In this case, TPNH is assumed to take part as a reductant. TPNH and oxygen might be involved in the formation of hydrogen peroxide, which in turn might be used for the N-demethylation

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10) M. Ishidate, Y. Hashimoto : This Bulletin, **7**, 108 (1959).
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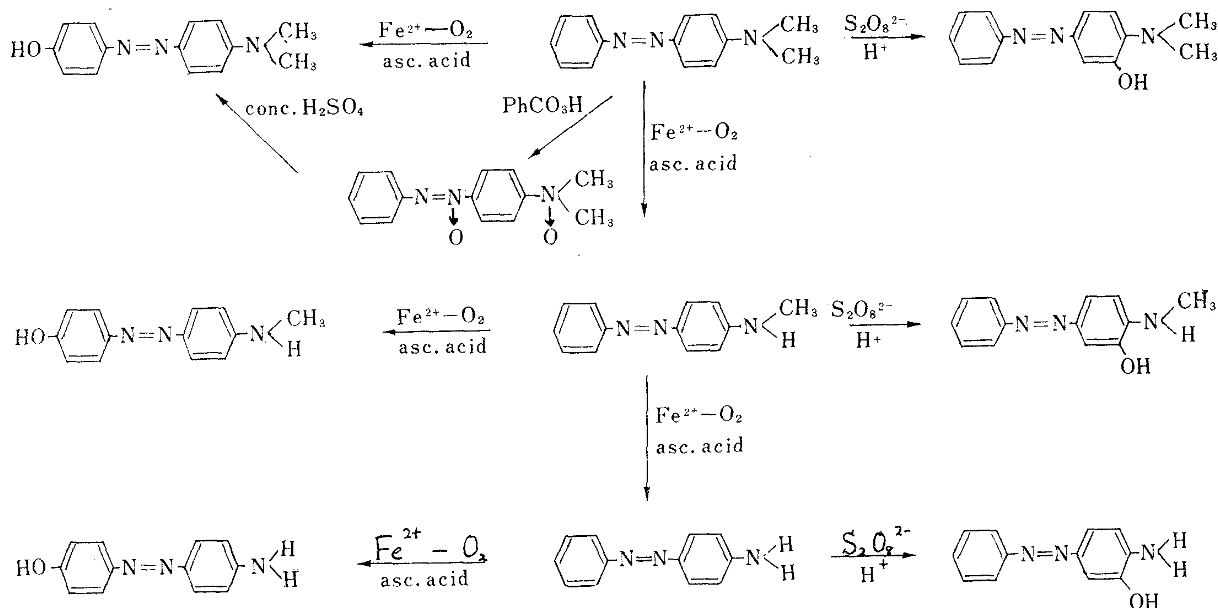
of DAB. The possibility that hydrogen peroxide was involved in the N-demethylase system was examined by adding hydrogen peroxide or glucose-glucose oxidase in place of TPNH. These results, however, indicated that hydrogen peroxide added or generated in the mixture was not involved in the N-demethylase system.[11]



asc. acid=ascorbic acid

Chart 1. Oxidative Pathway of DAB and its Derivatives

Among the oxidation methods mentioned above (Chart 1), the autoxidation system utilizes ascorbic acid as a reductant and requires free oxygen. Therefore, this system seemed worthy to be considered as an N-demethylase model.

Summary

Several model systems which catalyzed the oxidative N-demethylation of the carcinogenic N-methylaminoazo dyes were discussed. DAB, one of the most potent hepatocarcinogens, is not only demethylated to MAB, but hydroxylated to 4′-OH-DAB with hydrogen peroxide, peroxydisulfate, or free oxygen. Among these oxidations, the system consisting of the iron chelate of FDTA and ascorbic acid in the presence of free oxygen is considered to be a suitable N-demethylase model.

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