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Carcinogenic Azo Dyes. XII.[1] Detection of New Metabolites of Aminoazo Dyes by Rat Liver

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Metabolism of 3′-methyl-4-(dimethylamino)azobenzene (3′-Me-DAB) and 3′-methyl-4-(methylamino)azobenzene (3′-Me-MAB) by the rat liver was investigated by the use of a tracer technique. 3′-Me-DAB[5′-^{3}H] or 3′-Me-MAB[5′-^{3}H] was incubated with a 9000 ***g*** supernatant fraction at 37° in an aerobic condition for 30 min. The metabolites were extracted with benzene–acetone. The residual aqueous solution was applied to an Amberlite XAD-2 column and the metabolites were eluted with methanol. The metabolites in the extract or eluate were determined by the reverse isotope dilution analysis after separation by thin-layer chromatography.

The recovery of radioactivity in benzene–acetone extract after incubation of 3′-Me-DAB[5′-^{3}H] was 70.8±7.5%. About 99.5% of the radioactivity extracted was identified with the N-demethylated, oxidized at 4′ position and ring methyl group, and azo-reduced metabolites and the substrate. On the other hand, 79.4% or 83.8% of radioactivity recovered in each eluate was identified with 3-aminobenzoic acid and 3-acetaminobenzoic acid. Accordingly, it was proved that the metabolites oxidized at the ring methyl group was also detected in the *in vitro* metabolism of 3′-Me-DAB or 3′-Me-MAB.

Keywords——aminoazo compounds; chemical carcinogen; tracer technique; metabolic activation; new metabolites of 3′-Me-MAB and 3′-Me-DAB; carcinogenic influence of ring methyl

3′-Methyl-4-(dimethylamino)azobenzene (3′-Me-DAB) and 3′-methyl-4-(methylamino)-azobenzene (3′-Me-MAB) are much more potent hepatocarcinogens than DAB or MAB.[3] Kadlubar *et al.*[4] reported that a two-step enzymic activation mechanism of N-hydroxylation of the amino group followed by sulfonation of the N-hydroxy function is involved in the carcinogenic action of aminoazo dyes. However, the effect of ring methyl group on the carcinogenic action of aminoazo dyes has not been clearly proved. As a clue to elucidate the significance of ring methyl group, we investigated the metabolites of 3′-Me-DAB and 3′-Me-MAB in the rat bile and urine after the oral administration and reported that the oxidation products at the ring methyl group were detected as the new metabolites.[1,5] In the present work, metabolism of 3′-Me-DAB and 3′-Me-MAB by the rat liver was investigated by the tracer technique, using tritiated 3′-Me-DAB and 3′-Me-MAB.

3′-Me-DAB[5′-^{3}H] or 3′-Me-MAB[5′-^{3}H] was incubated with microsome suspension, and the substrate and metabolites were extracted with benzene–acetone mixture. The recovery of radioactivity in the extracts after incubation of 3′-Me-DAB[5′-^{3}H] was 70.8±7.5% (mean± SD) and that after incubation of 3′-Me-MAB[5′-^{3}H] 78.7±10.5%. 3-Aminobenzoic acid which was expected as a metabolite of the aminoazo dyes in the residual aqueous layers, was difficult to extract with any organic solvents from an aqueous solution. Therefore, the metabolites in the aqueous layers were separated by chromatography on the Amberlite XAD-2 using methanol (MeOH-1) and methanol containing HCl (MeOH-2) as solvent. The aqueous washing, MeOH-1, and MeOH-2 respectively contained 1.3, 26.6, and 0.3% of the radioactivity

1) Part XI: Y. Mori, T. Yamamoto, and K. Toyoshi, *Chem. Pharm. Bull.* (Tokyo), in press.
2) Location: *Mitahora-higashi 5-6-1, Gifu 502, Japan.*
3) J.A. Miller and E.C. Miller, *Adv. Cancer Res.*, **1**, 339 (1953).
4) F.F. Kadlubar, J.A. Miller, and E.C. Miller, *Cancer Res.*, **36**, 2350 (1976).
5) Y. Mori, T. Hori, K. Toyoshi, and M. Horie, *J. Pharm. Dyn.*, **1**, 192 (1978).

of 3′-Me-DAB[5′-^{3}H] added in the incubation mixture and 0.7, 20.2, and 0.4% of that of 3′-Me-MAB[5′-^{3}H]; the total recovery from the column was approximately 100% in these steps.

Each of benzene–acetone extracts was concentrated below 60° in a gentle stream of nitrogen and the substrate and metabolites were separated by thin-layer chromatography (TLC). Radiochromatograms of the metabolites in the extracts are given in Fig. 1 and 2. The metabolites in each peak were eluted from thin-layer plates with acetone or methanol and the radioactivity was counted by a liquid scintillation spectrometer. The relative ^{3}H activities are also shown in Fig. 1 and 2, and the metabolites in each peak were identified and determined quantitatively by the reverse isotope dilution analysis.[1)]

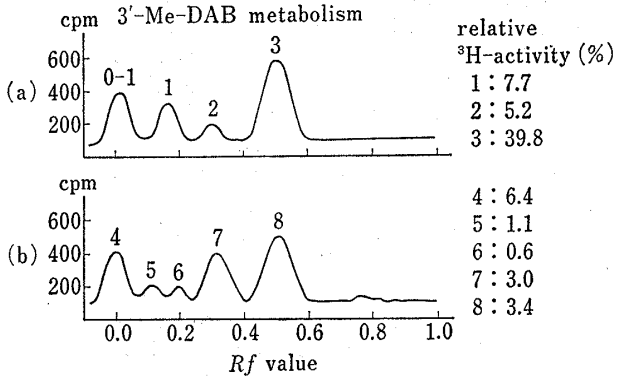

Fig. 1. Radiochromatogram of Benzene–acetone Extract (a) and Reradiochromatogram of Peak 0–1 (b)

Developing system
(a) benzene: petroleum benzine (2: 1),
(b) benzene: acetone (14: 1).

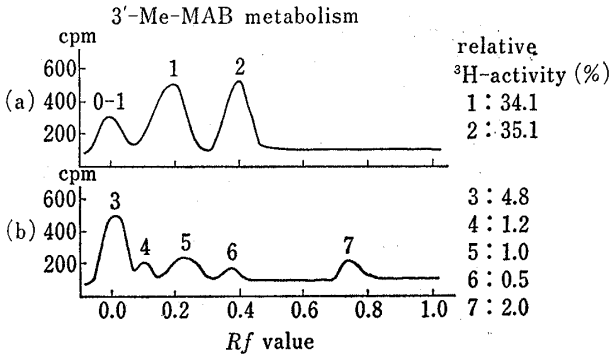

Fig. 2. Radiochromatogram of Benzene–acetone Extract (a) and Reradiochromatogram of Peak 0–1 (b)

Developing system
(a) benzene: petroleum benzine (2: 1),
(b) benzene: acetone (14: 1).

In a previous paper we reported that as the *in vitro* metabolites of 3′-Me-DAB, the azo-reduced, N-demethylated, and aryl hydroxylated metabolites were detected in peaks 1 to 8.[6)] The results of present study analyzing the metabolites oxidized at the ring methyl group revealed that 3′-CHO-DAB in peak 1, 3′-COOH-DAB, 3′-COOH-MAB, 3-aminobenzyl alcohol, and 3-acetamino benzyl alcohol in peak 4, 3′-CH_2OH-AB in peak 5, 3′-CH_2OH-MAB in peak 6, and 3′-CH_2OH-DAB in peak 7 were also detected as the minor metabolites in the extract, respectively. Consequently, about 99.5% of the radioactivity extracted in benzene–acetone layer was identified with the N-demethylated, oxidized at 4′-position and ring methyl group, and azo-reduced metabolites and the substrate. In the case of 3′-Me-MAB[5′-^{3}H] metabolism, the same metabolites were detected except the tertiary amines (Fig. 2).

Table I. Metabolites identified in the Eluate

Metabolite	Ratio (%) to eluate			
	3′-Me-DAB		3′-Me-MAB	
3-Aminobenzoic acid	50.6	79.4	73.7	83.8
3-Acetaminobenzoic acid	28.8		10.1	
3-Aminobenzyl alcohol	2.9		4.5	
3-Acetaminobenzyl alcohol	0.2		2.4	
3′-COOH-DAB	0.2		—	
3′-COOH-MAB	0.1		0.2	
Total	82.8		90.9	

6) S. Baba, Y. Mori, and K. Toyoshi, *Yakugaku Zasshi*, **92**, 1364 (1972).

For determination of the metabolites in the aqueous layers, carrier was added to a portion of the MeOH-1 eluate, the main fraction among the eluates, before separation by TLC and the metabolites were directly analyzed by the dilution method using the expected compounds. The ratios of the metabolites to each eluate are given in Table I. As expected, 3-aminobenzoic acid and its N-acetylated compound were main metabolites in the MeOH-1 eluate. Consequently, 82.8 or 90.9% of the radioactivity recovered in each eluate was identified as shown in Table I.

It is well known that the carcinogenic aminoazo dyes undergo oxidative N-demethylation, aryl hydroxylation, and reductive cleavage of the azo-linkage by the liver homogenate of a rat.[7,8] The present work revealed that these metabolites and, as the new metabolites, ones oxidized at the ring methyl group of 3′-Me-DAB or 3′-Me-MAB were detected in the metabolism by liver microsome suspension. It was previously reported that the metabolites in the aqueous layer, after extraction with benzene–acetone, were not proved at all.[6] However, the results of present study analyzing the metabolites in the aqueous layer also revealed that metabolites oxidized at the ring methyl group to carboxyl compounds were major ones in this fraction.

It was reported that 3′-halogenomethyl-DAB as the model compound for the ester of 3′-CH_2OH-DAB, which would lead to the carbonium ion formation, reacted with DNA *in vitro*.[9] We recognized that when an aminoazo dye was fed in the diet for 3 months to rats, 3′-CH_2OH-DAB was a more potent hepatocarcinogen than 3′-Me-DAB, while 3′-COOH-DAB was not (unpublished data). Therefore, it is suggested that the hydroxylation of ring methyl group of the carcinogenic aminoazo dyes may be one of the metabolic activation reactions.

Experimental

Male Wistar rats weighing 180—200 g were killed by decapitation. Strictly weighed pieces of the liver were homogenized in 3 volumes of cold 1.15% KCl in a crushed ice. 3′-Me-DAB[5′-^{3}H] or 3′-Me-MAB[5′-^{3}H] was incubated with 9000 ***g*** supernatant fraction at 37° in an aerobic condition for 30 min. A crude microsome fraction from 25% liver homogenate was prepared by centrifugation at 9000×***g*** for 30 min at 0°. The incubation mixture contained 1 μmol of 3′-Me-DAB[5′-^{3}H] or 3′-Me-MAB[5′-^{3}H] in 0.1 ml C_2H_5OH, 100 μmol of phosphate buffer (pH 7.4), 5 μmol of nicotinamide, 0.6 μmol of NADP, 0.6 μmol of NAD, 1.5 μmol of glucose-6-phosphate, 1.5 μmol of $MgCl_2$, and 2 ml of microsome suspension (corresponding to 0.65 g liver). The substrate and metabolites were extracted with benzene–acetone (1: 1, v/v), which was then concentrated under a gentle stream of nitrogen. The residue was spotted on a silica gel plate which was developed with benzene–petroleum benzine (2: 1, v/v), followed with benzene–acetone (14: 1). Radioactivity scan of the chromatograms was made with an Aloka TLC-101 scanner. The metabolites in each peak of scannograms obtained were eluted from the thin-layer plate with acetone or MeOH, and the radioactivity in each eluate was counted by a liquid scintillation spectrometer, Aloka LSC-651. Each metabolite was identified and determined quantitatively by the reverse isotope dilution analysis as previously reported.[1,8]

For determination of the metabolites in residual aqueous solution, this solution was processed on a column (bed volume, 2.5×20 cm) of Amberlite XAD-2 which was washed with 10 volumes of distilled water. After applying the aqueous solution, 200 ml of water was run into the column, and 250 ml of methanol (MeOH-1) and 150 ml of methanol containing 5% HCl (MeOH-2) were used to elute the polar metabolites. Carrier was then added to a portion of the eluate, and each eluate was concentrated *in vacuo* before separation by chromatography. The material obtained was recrystallized to a constant specific radioactivity.

7) J.A. Miller, *Cancer Res.*, **30**, 559 (1970); H. Terayama, "Methods in Cancer Research," vol. 1, ed. by H. Busch, Academic Press Inc., New York, 1967, p.399
8) Y. Mori, K. Toyoshi, S. Baba, and Y. Kasuya, *Chem. Pharm. Bull.* (Tokyo), **24**, 2279 (1976).
9) A. Dipple, *J. Chem. Soc. Perkin Trans.*, **1**, 447 (1972).