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Carcinogenic Azo Dyes. XI.[1] Analysis of Biliary and Urinary Metabolites of 3′-Methyl-4-(methylamino)azobenzene in Rat

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Metabolites of 3′-methyl-4-(methylamino)azobenzene (3′-Me-MAB) in the rat bile and urine were investigated by use of a tracer technique. ^{3}H-3′-Me-MAB in cottonseed oil was administered orally by a stomach tube. The dye metabolites in the bile and urine collected during 24 hr after the administration were hydrolyzed with β-glucuronidase/arylsulfatase. The hydrolyzed metabolites were then extracted with chloroform or separated by chromatography on an Amberlite XAD-2 using methanol as solvent. The metabolites in the chloroform extract or methanol eluate were identified by the reverse isotope dilution analysis, after or before separation by thin-layer chromatography.

The N-demethylated, aryl hydroxylated, and their azo-reduced products were detected in the bile, in addition to the products oxidized at the ring methyl group as the new metabolites of 3′-Me-MAB. On the other hand, the metabolites retaining the azo-linkage were hardly excreted in urine. Instead, 3-aminobenzoic acid, 3-amino-6-hydroxytoluene, and their N-acetylated products were major metabolites in urine. The present results indicate that the metabolism for 3′-Me-MAB in the rat involves oxidation at the ring methyl group. Effect of the ring methyl group on the carcinogenic action of aminoazo dyes is also discussed.

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

Introduction

Hepatocarcinogenicity of several aminoazo dyes is well known.[3] Kadlubar *et al.* 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.[4]

On the other hand, aminoazo dyes substituted with a methyl group, such as 3′-methyl-4-(methylamino)azobenzene (3′-Me-MAB) and 3′-methyl-4-(dimethylamino)azobenzene (3′-Me-DAB), are much more potent hepatocarcinogens than MAB or DAB.[3] However, the effect of ring methyl group on the carcinogenic potency of aminoazo dyes and the metabolites of 3′-Me-MAB in the rat have not been clarified. As a clue to elucidate the significance of ring methyl group in the carcinogenesis by 3′-Me-MAB, we investigated metabolites of 3′-Me-MAB in the rat bile after its oral administration by the ion cluster technique and reported that the oxidation products at the ring methyl group were detected as the new metabolites.[5] In the present work, metabolites of 3′-Me-MAB in the rat bile and urine were quantitatively analyzed by the reverse isotope dilution method, using tritiated 3′-Me-MAB.

Experimental

Chemicals——3′-Me-MAB[5′-^{3}H] were synthesized by the method described in a previous paper of this series.[5] Compounds used for the dilution analysis as carrier were also synthesized in our laboratory.[6]

1) Part X: Y. Mori, T. Hori, and K. Toyoshi, *Gann*, **69**, 757 (1978).
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, M. Horie, and K. Toyoshi, *Radioisotopes*, **28**, 72 (1979)
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Metabolites of 3′-Me-MAB in Rat Urine——Male Wistar rats weighing 180—220 g were orally administered 46 mg/kg of 3′-Me-MAB[5′-^{3}H] in 1 ml of cottonseed oil by a stomach tube. Urine was collected for 24 hr after the administration. The urine was adjusted to pH 4.5 with 200 ml of acetate buffer and incubated with β-glucuronidase (1.5 U; EC 3.2.1.31)/arylsulfatase (0.8 U; EC 3.1.6.1) (Boehringer Mannheim) for 24 hr at 37°. The hydrolyzed urine, after neutralization with NaOH and saturation with NaCl, was processed on a column (2.5×20 cm) of Amberlite XAD-2 which was washed with 10 volumes of distilled water. After applying the urine, 500 ml of water, 450 ml of methanol (MeOH-1), and 250 ml of methanol containing 5% HCl (MeOH-2) were run successively. The MeOH-1 or -2 eluate was reasonably divided, and each carrier which was expected as the metabolite of 3′-Me-MAB in the urine was added to the portion of eluate, which was then concentrated *in vacuo*. The material obtained was separated by thin-layer chromatography and repeatedly recrystallized until a constant specific radioactivity was attained.

Metabolites of 3′-Me-MAB in Rat Bile——Rats were anesthetized by an intraperitoneal injection of 5% sodium pentobarbital and a polyethylene tube was inserted into the bile duct. A mixture of 46 mg/kg of 3′-Me-MAB[5′-^{3}H] and 1 ml of cottonseed oil was administered into the stomach of each rat through a catheter and the bile was collected for 1 to 35 hr. The unconjugated metabolites were extracted with $CHCl_3$ ($CHCl_3$-1) from the 24 hr bile and the remaining bile was re-extracted with $CHCl_3$ ($CHCl_3$-2) after hydrolysis with β-glucuronidase/arylsulfatase and neutralization with NaOH. The residual aqueous layer was saturated with NaCl and applied on a column of Amberlite XAD-2 as described in the urine experiment. The organic extracts ($CHCl_3$-1 and -2) were washed with water, dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and subjected to separation by chromatography.

Thin-layer chromatography (TLC) of $CHCl_3$-1 and -2 was run routinely to determine the location of 3′-Me-MAB and its metabolites. The metabolites were spotted on silica gel (Wakogel B-5F) plates (20×20 cm) and developed sequentially with the solvent systems consisting of benzene–petroleum benzine (2:1, v/v), benzene–acetone (14:1), and $CHCl_3$–MeOH (8:1). Radioactivity scan of the chromatograms was made with an Aloka TLC-101 scanner. The metabolites in each peak of scannograms obtained were eluted from thin-layer plates with acetone or methanol, and the radioactivity in each eluate was counted by a liquid scintillation spectrometer, Aloka LSC-651. Each metabolite in the eluates was identified and determined quantitatively by the reverse isotope dilution analysis as previously reported.[7]

Results

Metabolites in Rat Bile

Figure 1 shows the biliary excretion of radioactivity after oral administration of 3′-Me-MAB[5′-^{3}H]. The peak of the excretion rate appeared at 7 hr under the conditions used, and the cumulative recovery at 24 hr reached 98% of the total activity excreted during 35 hr. Therefore, the 24-hr bile was used for the assay of metabolites in the rat bile. The bile collected during 24 hr was about 17—20 ml. Of 4.72×10^8 dpm administered, the 24-hr bile contained 1.36×10^8 dpm or 28.7±3.4% (mean±SD) of the administered activity. The metabolites were extracted with chloroform and separated by chromatography on the Amberlite XAD-2 using methanol as solvent. The $CHCl_3$-1, $CHCl_3$-2, aqueous washing, MeOH-1, and MeOH-2 respectively contained 23.1, 17.0, 7.9, 37.3, and 9.7% of the excreted activity; the total recovery from the 24-hr bile was about 95% in these steps. This loss seems to be due to adsorption on the resin.

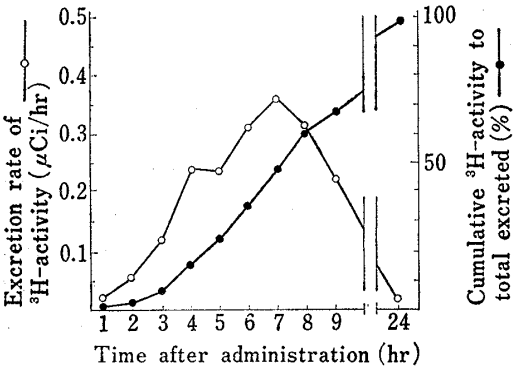

Fig. 1. Biliary Excretion of the Metabolites after Oral Administration of ^{3}H-3′-Me-MAB

The $CHCl_3$-1 and -2 were concentrated *in vacuo*, and the metabolites were separated by TLC. Radiochromatograms of the metabolites in $CHCl_3$-1 and -2 are given in Fig. 2. The main radioactive peaks recognized in the scannograms of $CHCl_3$-1 and -2 were five and

7) Y. Mori, K. Toyoshi, S. Baba, and Y. Kasuya, *Chem. Pharm. Bull.* (Tokyo), **24**, 2279 (1976).

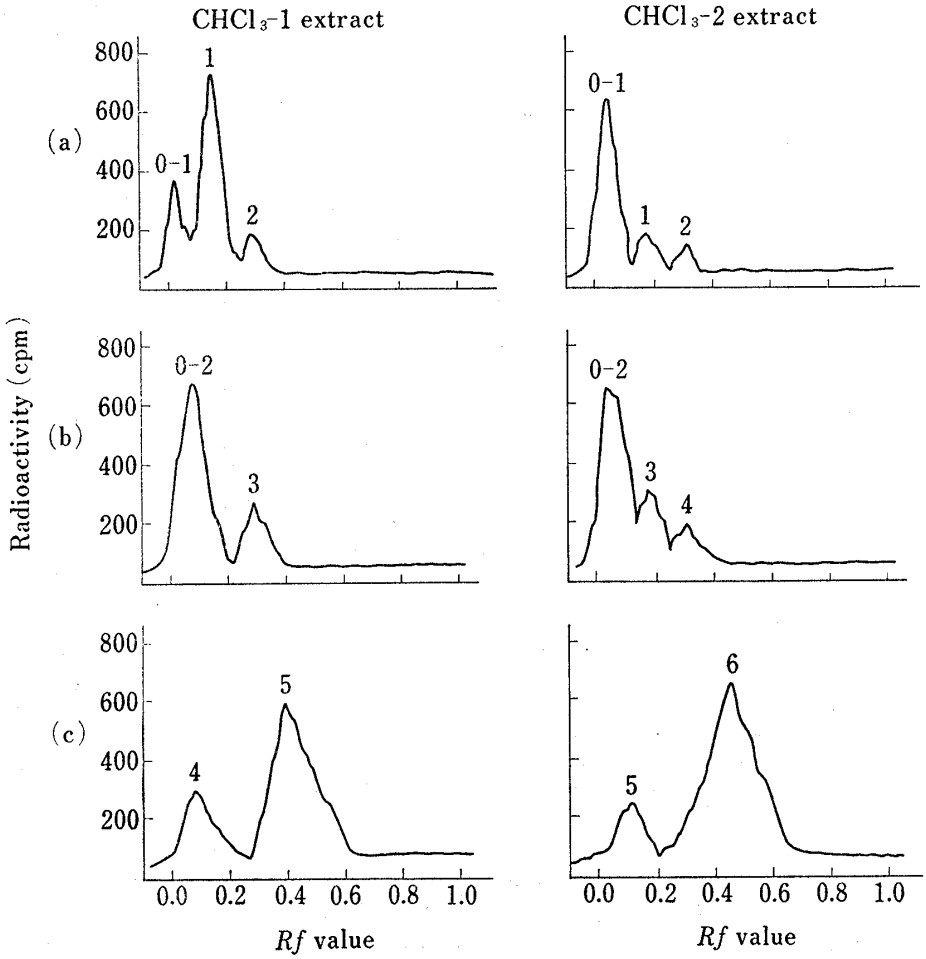

Fig. 2. Radiochromatograms of $CHCl_3$ Extracts from Bile (a) and Reradiochromatograms of Peak 0—1 (b) and 0—2 (c)

Developing system
(a) benzene: petroleum benzine (2: 1),
(b) benzene: acetone (14: 1),
(c) chloroform: methanol (8: 1).

six, respectively. The metabolites in each peak were eluted from thin-layer plates with acetone or methanol. The relative radioactivities counted by a liquid scintillation spectrometer and the results of the dilution analysis with each peak are summarized in Tables I and II.

The N-demethylated metabolite (3′-methyl-4-aminoazobenzene=3′-Me-AB), the administered substance (3′-Me-MAB), and their azo-reduced metabolites (3-aminotoluene and 3-acetaminotoluene) formed 78.72% of the radioactivity extracted in the $CHCl_3$-1 which contained the unconjugated metabolites, while the metabolites oxidized at the benzene ring or ring methyl group were minor ones. Consequently, 87.2% of the activity extracted in the $CHCl_3$-1 was identified as shown in Table I. In the $CHCl_3$-2, 3′-Me-AB and 3′-Me-MAB formed 8.09% of the activity extracted. The aryl hydroxylated metabolites (3′-Me-4′-OH-AB, 3′-Me-4′-OH-MAB, and 3′-Me-4′-OH-AB-NAc) and their azo-reduced ones (3-amino-6-hydroxytoluene and 3-acetamino-6-hydroxytoluene) respectively formed 25.57 and 7.27% of the activity. The metabolites oxidized at the ring methyl group were also detected as minor ones and their azo-reduced metabolites as the main ones. Consequently, 58.5% of the activity extracted in the $CHCl_3$-2 was identified as shown in Table II. In the conjugated metabolites, the ratio of the sulfates to glucuronides was about 3: 5, based on the recovery experiments using β-glucuronidase and arylsulfatase separately.

Table I. Metabolites identified in $CHCl_3$ Extract obtained from the Bile of Rat given 3′-Me–MAB[5′–^{3}H]

Peak	Relative ^{3}H activity [A]$^{a)}$	Metabolite	Percentage to peak [B]	$A \times \frac{B}{100}$ (%)$^{b)}$	
1	73.5	3′-Me–AB	85.8	63.06	
		3-Aminotoluene	13.2	9.70	78.72
2	5.7	3′-Me–MAB	26.8	1.53	
3	8.8	3-Acetaminotoluene	50.3	4.43	
		3′-Me–MAB–NAc	29.8	2.62	
		3′-CH_2OH–MAB	5.0	0.44	
		3′-CH_2OH–AB	1.1	0.10	
		3′-Me-4′-OH–MAB	0.6	0.05	
		3′-Me-4′-OH–AB	1.2	0.11	
4	4.6	3-Aminobenzoic acid	38.8	1.78	
		3-Acetaminobenzoic acid	18.6	0.86	
		3′-COOH–MAB	6.7	0.31	
5	7.4	3-Amino-6-hydroxytoluene	10.9	0.81	
		3-Acetamino-6-hydroxytoluene	8.5	0.63	
		3-Aminobenzyl alcohol	4.6	0.34	
		3-Acetaminobenzyl alcohol	4.4	0.33	
		3′-Me-4′-OH–AB–NAc	1.4	0.10	
Total	100.0			87.20	

The values are the percentages to the total ^{3}H activity extracted in $CHCl_3$-1.
a) The radioactivity of peak in the scannograms shown in Fig. 2.
b) The radioactivity of each metabolite.

Table II. Metabolites identified in $CHCl_3$ Extract obtained from the Hydrolyzed Bile of Rat after $CHCl_3$-1 Extraction

Peak	Relative ^{3}H activity [A]$^{a)}$	Metabolite	Percentage to peak [B]	$A \times \frac{B}{100}$ (%)$^{b)}$
1	12.1	3′-Me–AB	49.6	5.99
		3-Aminotoluene	0.2	0.02
2	3.3	3′-Me–MAB	63.7	2.10
3	24.5	3′-Me-4′-OH–AB	21.9	5.36
		3′-CH_2OH–MAB	2.7	0.66
		3′-CH_2OH–AB	3.0	0.74
4	5.7	3′-Me-4′-OH–MAB	19.0	1.08
5	16.8	3-Aminobenzoic acid	41.9	7.04
		3-Acetaminobenzoic acid	34.7	5.83
		3′-COOH–MAB	14.0	2.35
6	32.2	3′-Me-4′-OH–AB–NAc	59.5	19.13
		3-Amino-6-hydroxytoluene	12.4	3.99
		3-Acetamino-6-hydroxytoluene	10.2	3.28
		3-Aminobenzyl alcohol	1.5	0.48
		3-Acetaminobenzyl alcohol	1.4	0.45
Total	94.6			58.50

The values are the percentages to the total ^{3}H activity extracted in $CHCl_3$-2.
a) The radioactivity of peak in the scannograms shown in Fig. 2.
b) The radioactivity of each metabolite.

Table III summarizes the metabolites in the MeOH-1, the main fraction among the extracts and eluates. In this polar fraction, which contained the metabolites difficult to extract with chloroform, 3-aminobenzoic acid and 3-acetaminobenzoic acid were determined as the major metabolites, and about 100% of the radioactivity recovered in the eluate was identified as shown in Table III.

Table III. Biliary Metabolites of 3′-Me–MAB[5′–^{3}H] in the Eluate

Metabolite	Percentage to MeOH-1
3-Aminobenzoic acid	47.95
3-Acetaminobenzoic acid	34.18
3-Aminobenzyl alcohol	0.54
3-Acetaminobenzyl alcohol	1.88
3-Amino-6-hydroxytoluene	0.31
3-Acetamino-6-hydroxytoluene	13.13
3′–COOH–MAB	2.76
Total	100.75

Recovery of ^{3}H activity in MeOH-1 eluate is 37.3% of the excreted activity.

Metabolites in Rat Urine

Of 4.72×10^8 dpm administered, the 24-hr urine contained 57.5 ± 0.3% of the administered activity. The collected urine was enzymically hydrolyzed and the hydrolyzate was then submitted to chromatography on an Amberlite XAD-2 column. The aqueous washing, MeOH-1, and MeOH-2 respectively contained 6.0, 59.9, and 27.9% of the activity excreted in the urine; the radioactivity recovery in the chromatography was approximately 94%. The MeOH-1 and -2 eluates were concentrated *in vacuo* and the metabolites were separated by TLC. However, the peak recognized in the radiochromatograms of the metabolites in the eluates was mainly at the origin on TLC under the conditions used (Fig. 2); this result indicates that the metabolites retaining the azo-linkage were hardly excreted in the urine. Therefore, the MeOH-1 and -2 were directly analyzed by the dilution method using expected compounds. The composition of the metabolites in each of eluates (MeOH-1 and -2) are given in Table IV. The products oxidized at the ring methyl group formed approximately

Table IV. Metabolites identified in the Eluates obtained from the Hydrolyzed Urine of Rat given 3′-Me-MAB[5′–^{3}H]

Metabolite	Percentage to MeOH eluate		(%)[a]	
	MeOH-1	MeOH-2		
3-Aminobenzoic acid	29.00	22.32	23.57	
3-Acetaminobenzoic acid	34.76	6.03	22.50	48.38
3-Aminobenzyl alcohol	0.20	0.98	0.39	
3-Acetaminobenzyl alcohol	2.24	2.10	1.92	
3-Amino-6-hydroxytoluene	6.35	10.60	6.76	30.25
3-Acetamino-6-hydroxytoluene	19.95	41.50	23.49	
3-Aminotoluene	4.63	15.29	7.02	
3-Acetaminotoluene	1.03	1.04	0.90	
Total	98.16	99.86	86.55	

The radioactivity in MeOH-1 and MeOH-2 respectively were 59.9 and 27.9% recoveries.
a) The values are the percentages of metabolite taking ^{3}H activity excreted in urine as 100.

50% of the radioactivity excreted in the urine and the aryl hydroxylated ones, 30%. Moreover it was found that 86.55% of the total activity excreted in the urine was represented by the azo-reduced products.

Discussion

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

rats.[7-9] The results of present study analyzing the metabolites in the urine and bile revealed that these metabolites and, as the new metabolites, ones oxidized at the ring methyl group of 3′-Me-MAB, 3′-CH_2OH- and 3′-COOH-aminoazo dyes, were determined in the rat bile. Especially, in the urine, metabolites oxidized at the methyl group to carboxyl compounds were major rather than the metabolites retaining the methyl group. These results indicate that the metabolism of 3′-Me-MAB in the rat involves oxidation of the ring methyl group as shown in Fig. 3. Of these metabolites, 3′-Me-AB, 3′-Me-4′-OH–AB, 3′-Me-4′-OH–AB–

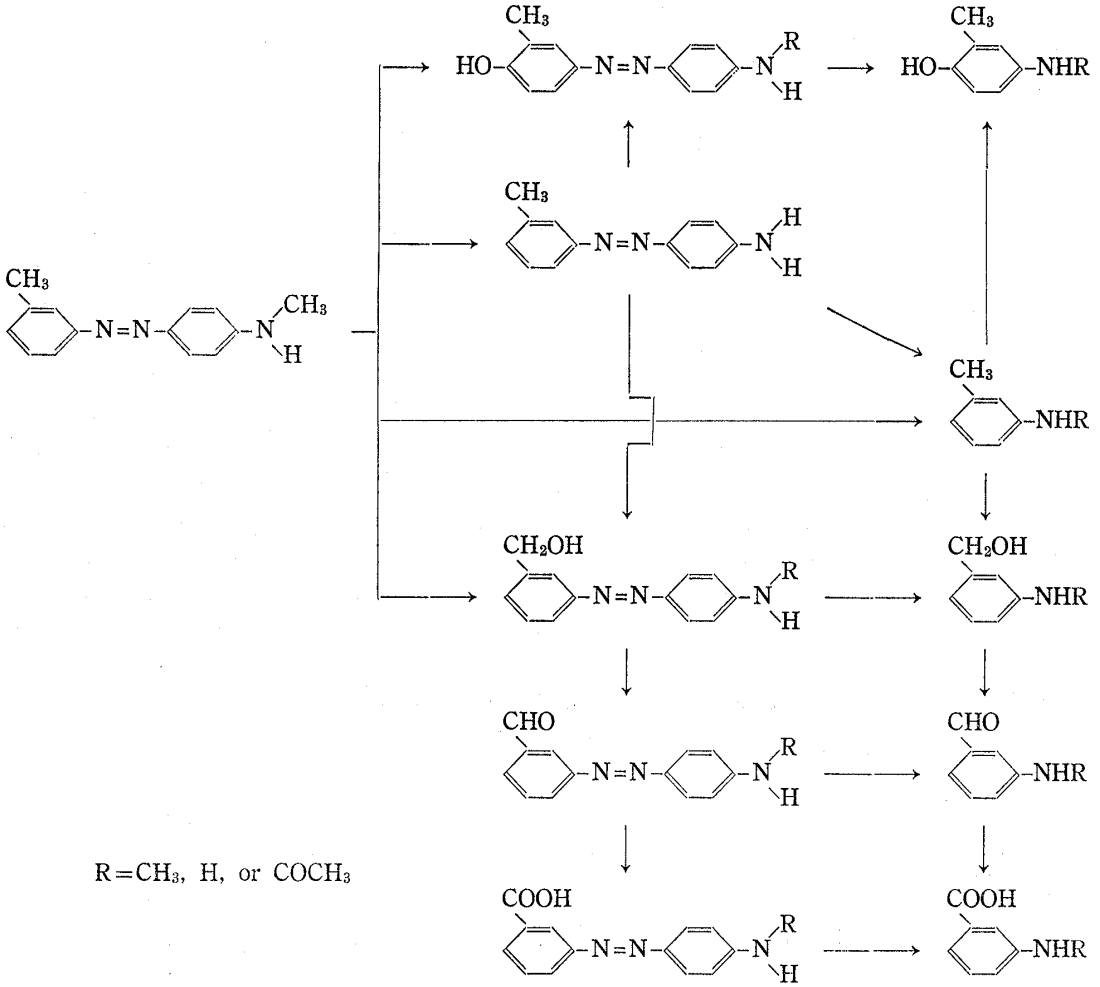

Fig. 3. Metabolic Pathway of 3′-Me-MAB in Rat

NAc, 3-aminotoluene, 3-amino-6-hydroxytoluene, 3-aminobenzoic acid, and their N-acetylated compounds were major metabolites in the rat bile (Tables I, II, and III) and 3-aminobenzoic acid, 3-amino-6-hydroxytoluene, and their N-acetylated compounds in the urine (Table IV).

Covalent binding of the carcinogens to cellular macromolecules *in vivo* is the most likely first step of their carcinogenic action, and one of the characteristics of proximate and ultimate carcinogens gives their reactivity *in vitro*.[8] 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*.[10] Lin investigated the carcinogenicity of a number of azo dyes disubstituted with several amino groups, and some disubstituted azo dyes were

8) J.A. Miller, *Cancer Res.*, **30**, 559 (1970).
9) H. Terayama, "Methods in Cancer Research," ed. H. Busch, Vol. 1, Academic Press Inc., New York, 1967, p. 399.
10) A. Dipple, *J. Chem. Soc. Perkin Trans.*, **1**, 447 (1972).

found to be more carcinogenic (2—4 times) than the original aminoazo dye.[11] 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, whereas 3′–COOH–DAB was not.[12] Accordingly, it was suggested that the hydroxylation of ring methyl group of the carcinogenic aminoazo dyes was one of the metabolic activation reactions.

11) J.-K. Lin, *Proc. Nat. Sci. Council* (Taiwan), **No. 8**, 101 (1974).
12) Y. Mori, H. Nagai, T. Hori, K. Toyoshi, and A. Koda, *Gann*, "submitted."