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Studies on Carcinogenic Azo Dyes. VII.[1] Changes in the Hepatic Activities of 3′-Me-DAB Metabolism by Rat, Mouse, and Hamster during the Repeated Administration of 3′-Me-DAB[2]

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The metabolic pattern of 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) in the rat liver through the course of hepatic carcinogenesis by 3′-Me-DAB and the relationship between the hepatic activity for 3′-Me-DAB metabolism and species difference observed in the carcinogenic activity of the dye were investigated. The hepatic activity for 3′-Me-DAB metabolism with the liver of rats, mice, and hamsters fed a diet containing 3′-Me-DAB was measured by the tracer technique. By the repeated administration of 3′-Me-DAB, the hepatic azo-reduction activity of hamsters was depressed but their N-demethylation activity was conversely promoted throughout the observation period. Consequently, the total conversion of 3′-Me-DAB to metabolites in the liver of hamsters fed the dye remained almost the same as that of the control. In the rat liver, the most sensitive organ for carcinogenesis, 3′-Me-DAB administration had a marked effect on 3′-Me-DAB-metabolizing activity, and the azo-reduction activity was significantly depressed by the feeding of 3′-Me-DAB diet already from 6 hr later throughout the experimental period. In contrast, the N-demethylation reaction of 3′-Me-DAB to 3′-methyl-4-methylaminoazobenzene, a proximate carcinogen, was enhanced in the rat and mouse liver by their treatment only at the early period when the formation of protein-bound dye in the liver rose to a maximum peak by 3′-Me-DAB administration. In the rat hepatic tumors formed by the administration of 3′-Me-DAB for 3—4 months, all the activities for 3′-Me-DAB metabolism were notably decreased. Thus, it was found that the hepatic activities for 3′-Me-DAB metabolism were specifically affected by 3′-Me-DAB administration in all the three species, in which 3′-Me-DAB is carcinogenic or not.

Introduction

Miller[4] suggested that aromatic amine and amide carcinogens might be precarcinogens which are metabolized in the host and converted into carcinogenic and reactive structures (proximate carcinogens), and that ultimate carcinogen of the aminoazo dyes in the rat liver is an ester of their N-hydroxylated metabolites.

The aminoazo dye carcinogenesis shows a specificity in regard to species and an organ; under most conditions only two species, the rat and the mouse, have proved susceptible to

1) Part VI: Y. Mori, K. Toyoshi, and S. Baba, *Chem. Pharm. Bull.* (Tokyo), **24**, 500 (1976).
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4) J.A. Miller, *Cancer Res.*, **30**, 559 (1970).

hepatic carcinogenesis by aminoazo dyes, and the liver of a rat is the most sensitive organ.[5] 4-Dimethylaminoazobenzene and 3′-methyl-4-dimethylaminoazobenzene[6] (3′-Me-DAB) undergo oxidative N-demethylation, aryl hydroxylation, and reductive cleavage of the azo linkage by the liver homogenate of a rat. It has been reported that the factors retarding or accelerating aminoazo dye carcinogenesis have much influence on these metabolic activities in the rat liver,[7–9] and that rat liver tumors produced by these carcinogens do not metabolize an appreciable amount of aminoazo dyes.[10,11]

Therefore, it was of interest to clarify the metabolic pattern of a carcinogen in the rat liver through the course of carcinogenesis by aminoazo dyes, and the relationship between the metabolic activity and the species difference observed in carcinogenic activity of the aminoazo dye. In previous papers,[6,12] we reported that the hepatic activities for 3′-Me-DAB metabolism in rats, mice, and hamsters may be more directly determined by the use of a tritiated substrate than by a colorimetric method, and that this tracer technique is a useful method for studying metabolism by the liver after a repeated administration of a chemical compound to animals, since the hepatic activity can be measured without the effect of compounds accumulated in the liver by a pretreatment. We report in this paper, the effect of 3′-Me-DAB administration for 1—17 weeks on the hepatic activity of the metabolism of this carcinogen in rats, mice, and hamsters, in which 3′-Me-DAB is not carcinogenic, by the use of this tracer technique.

Experimental

Animals and Diet——Wistar strain rats, dd strain mice, and golden hamsters, weighing 140—150 g, 18—20 g, an d 80—85 g, respectively, at the start of the experiment were used. Animals were given the MF cube diet (Oriental Yeast Ind. Tokyo) for 1 week, and all the male animals were randomly divided into two groups. One group of animals serving as a control, received the basal diet MF, and the other group was fed the MF containing 0.06% 3′-Me- DAB, also supplied from Oriental Yeast Ind. The diet and water were given freely throughout the experiment. The weight of animals in each group up to about 3 months and the amount of the diet consumed in the early stage of feeding were measured.

Determination of Hepatic Activity of 3′-Me-DAB Metabolism——In each experimental group, the animals were killed by decapitation between 1 and 17 weeks after the start of the experiment. Strictly weighed pieces of the liver were homogenized in 1.15% KCl solution with a Potter homogenizer having a Teflon pestle, in crushed ice, and 20% liver homogenate thus prepared was used as the enzyme solution. For estimation of the enzyme activity, the incubation mixture described by Miller, *et al.*[13] was slightly modified. A metabolic system contained 0.5 ml of 0.1 M phosphate buffer (pH 7.4), 0.1 ml of 0.1% NADP, 0.1 ml of 0.1% NAD, 0.2 ml of 0.6 M nicotinamide, 0.1 ml of 0.1 M glucose 6-phosphate, 0.1 ml of 0.1 M $MgCl_2$, 0.6 ml of 1.15% KCl, 1 ml of the homogenate, and 0.569 μmol of 3′-Me-DAB[5′-^{3}H][14] in 0.05 ml EtOH, which was added last. The total volume of this system was 2.75 ml. The incubation flask was shaken at 37° in an aerobic condition for 15 min. The rate of total conversion of 3′-Me-DAB to metabolites was adequately linear for 20 min under the above conditions. At the 17th week of the dye feeding rats tumor liver and liver tissues freed from tumor from dye-fed rats were prepared for the enzyme assay. All the metabolic reaction was stopped by the addition of about 5 ml of benzene and acetone mixture (1: 1, v/v), which simultaneously extracted the substrate and the metabolites in the reaction mixture. The extractions were repeated with the same solvent until tritium activity was not detected in the upper organic solvent layer, usually 3 or 4 times. Each of the benzene–acetone extracts was concentrated below 60° in a gentle stream of nitrogen. The residue was spotted on a silica gel plate (5 × 20 cm) which was developed with benzene–petro. benzine (2: 1, v/v), followed with benzene–acetone (7: 1, v/v).[6] Each metabolite was eluted

5) E.C. Miller and J.A. Miller, *J. Natl. Cancer Inst.*, **15**, 1571 (1955).
6) S. Baba, Y. Mori, and K. Toyoshi, *Yakugaku Zasshi*, **92**, 1364 (1972).
7) E.C. Miller, J.A. Miller, R. Brown, and J.C. Macdonald, *Cancer Res.*, **18**, 469 (1958); J.W. Cramer, J.A. Miller, and E.C. Miller, *J. Biol. Chem.*, **235**, 250 (1960).
8) K. Takamiya, S.H. Chen, and H. Kitagawa, *Gann*, **64**, 366 (1970); R. Kato, *Jpn. J. Pharmacol.*, **17**, 181 (1967).
9) Y. Yamane and K. Sakai, *Gann*, **64**, 563 (1973); *Chem. Pharm. Bull.* (Tokyo), **23**, 1440 (1975).
10) C.J. Kensler, J.W. Magill, and K. Sugiura, *Cancer Res.*, **7**, 95 (1947).
11) M. Matsumoto and H. Terayama, *Gann*, **51**, 255 (1960).
12) K. Toyoshi, Y. Mori, and S. Baba, *Yakugaku Zasshi*, **93**, 1554 (1973).
13) A.H. Conney, R.R. Brown, J.A. Miller, and E.C. Miller, *Cancer Res.*, **17**, 628 (1957).
14) S. Baba, Y. Mori, M. Iwao, and S. Iwahara, *Yakugaku Zasshi*, **89**, 1158 (1969).

from the thin-layer plate with 3 ml of acetone, and was identified and determined quantitatively by the reverse isotope dilution analysis as previously reported.[6] The rate of each metabolic reaction was expressed as follows: For azo-reduction, 3-toluidine and 3-acetamidotoluene produced in nmol/200 mg liver/15 min; for N-demethylation, 3′-methyl-4-methylaminoazobenzene (3′-Me-MAB) and 3′-methyl-4-aminoazobenzene (3′-Me-AB) produced in nmol/200 mg liver/15 min; and for aryl hydroxylation, 3′-methyl-4′-hydroxy-4-dimethylaminoazobenzene (3′-Me-4′-OH-DAB) produced in nmol/200 mg liver/15 min.

Results

Effect of 3′-Me-DAB Feeding on Body Weight and Diet Consumption

The growth curve of animals and amount of the diet consumed in each group are shown in Figs. 1 and 2. There was no significant difference in the growth rate and the diet consumption of hamsters and mice between the two groups, but increase in the body weight of rats was very slow in the dye-fed group throughout the experiment, and diet consumption of rats fed with the dye was lower than that of the control rats. Therefore, it seems likely that the markedly

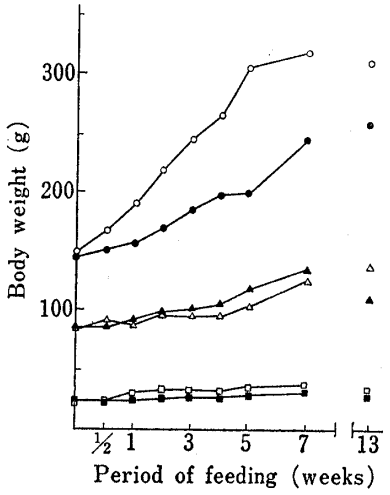

Fig. 1. Growth Curve of Animals after Feeding of Experimental Diet

basal diet; rats —○—, mice —□—, hamsters —△—; 0.06% 3′-Me-DAB containing diet; rats —●—, mice —■—, hamsters —▲—

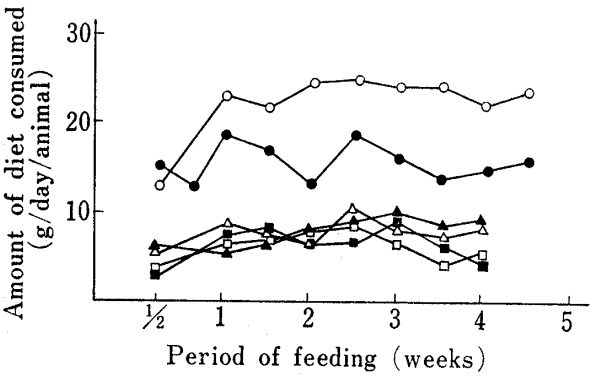

Fig. 2. Diet Consumption during Repeated Feeding

Symbols are the same as those in Fig. 1.

restrained increase of body weight in the rats treated with 3′-Me-DAB was not only due to the toxicity of this carcinogen but to undernourishment of the rats. This observation with rat is in agreement with a previous work in which the growth rate and diet consumption were measured after feeding 0.06% DAB or 3′-Me-DAB diet for 1 to 20 weeks in male Donryu[8] or female Wistar[9] rats.

Total Conversion of 3′-Me-DAB to Metabolites

In any experiment, the radioactivity recovered in the benzene–acetone layer from the incubation mixture was approximately constant (85%) by the methods of extraction used in this experiment. Fig. 3 shows the total conversion of 3′-Me-DAB to metabolites with the liver homogenate of hamsters, mice, and rats in term of the amount of 3′-Me-DAB metabolized (%) by each incubation.

The hepatic activity of hamsters fed with the dye was almost the same level as that of the control group except for a slight decrease at the 3rd week. That of mice in the dye-fed group was slightly higher or the same level as that of the control up to 3 weeks of feeding, but after the 5th week, approximately 5—10% lower than that of the control ($p<0.01$). By the

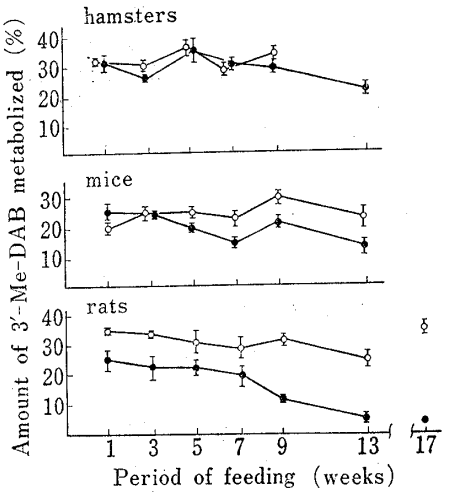

Fig. 3. Total Conversion of 3'-Me-DAB to Metabolites by Liver Homogenate

Each group contained 3—5 animals, and the values are their mean±SD.
basal diet (○), 0.06% 3'-Me-DAB diet (●)

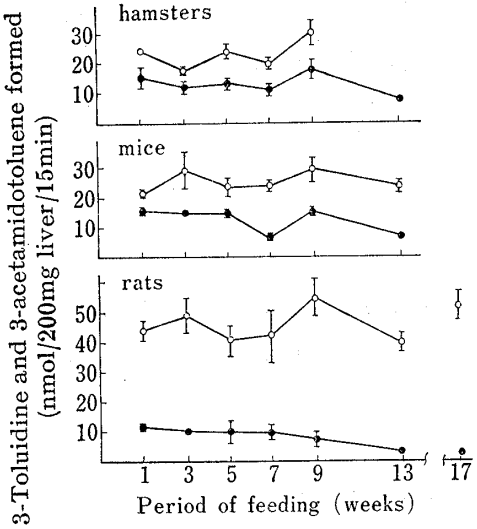

Fig. 4. Effect of 3'-Me-DAB Feeding on Hepatic Azo-reduction Activity

Each group contained 3—5 animals, and the values are their mean±SD.
basal diet (○), 0.06% 3'-Me-DAB diet (●)

3'-Me-DAB administration, the amount of 3'-Me-DAB metabolized by a rat liver was approximately 10% lower during the 1st to 7th week than that of the control ($p<0.01$) and it was remarkedly reduced especially after the 9th week when tumor in the liver was found in recognizable size.

Effect of 3'-Me-DAB Treatment on 3'-Me-DAB Azo-reduction Activity

The hepatic activity for azo-reduction decreased in all three species by the administration of 3'-Me-DAB throughout the experimental course, as shown in Fig. 4. However, the activity was depressed by the feeding to the greatest extent in rats among the three species. The amount of azo-reduced products in the dye-fed group of rats was only one-fourth or one-fifth of that of the control in each experimental period up to the 9th week. Depression of the activity in the rat liver was due to a great decrease in the amount of 3-toluidine produced, but the amount of 3-acetamidotoluene produced did not exhibit a significant difference from that in the control. Depression of hepatic activity in mice of the dye-fed group seems due to the decrease of production of both 3-toluidine and 3-acetamidotoluene, while that in hamsters is due to the decrease in the amount of 3-acetamidotoluene produced in the azo-reduction.

Effect of 3'-Me-DAB Treatment on N-Demethylation of 3'-Me-DAB

In Fig. 5, hepatic N-demethylation activity in each animal is compared with that of the control. The liver in the dye-fed hamsters showed a constantly higher activity throughout the observation period, and the metabolic reaction of 3'-Me-DAB to both 3'-Me-MAB and 3'-Me-AB was elevated by the azo-dye feeding. The mice in the dye-fed group tended to show a higher level than the control during the 1st to 3rd week, and after the 5th week up to the 9th week, feeding of 3'-Me-DAB diet had no marked effect on the activity. In the rat liver, N-demethylation reaction of 3'-Me-DAB to 3'-Me-MAB was enhanced in the dye-fed group in the 1st week, and coversely the activity was depressed to a greater extent after the 9th week.

Effect of 3'-Me-DAB Treatment on 3'-Me-DAB Aryl Hydroxylation Activity

As shown in Fig. 6, the hepatic activity for aryl hydroxylation in hamsters and mice, in the range of 3—5 nmol and 0.9—2 nmol/200 mg liver/15 min, respectively, were not apparently affected by 3'-Me-DAB administration throughout the experimental period and,

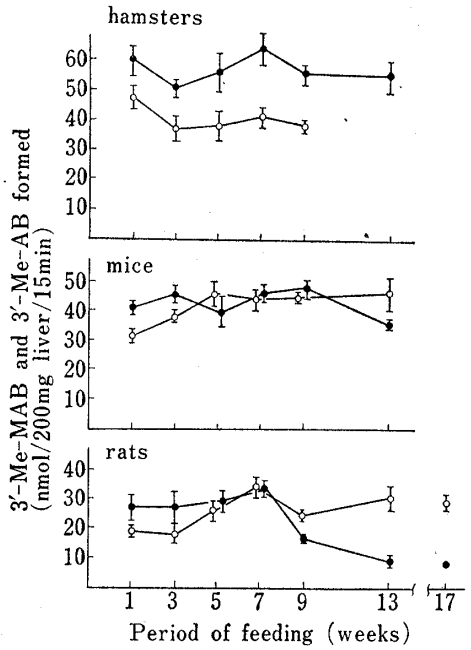

Fig. 5. Effect of 3'-Me-DAB Feeding on Hepatic N-Demethylation Activity

Each group contained 3—5 animals, and the values are their mean ± SD.
basal diet (○), 0.06% 3'-Me-DAB diet (●)

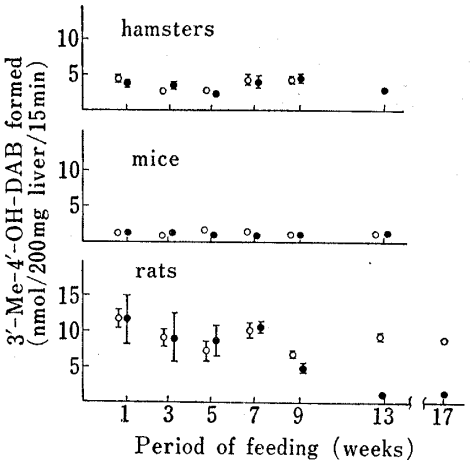

Fig. 6. Effect of 3'-Me-DAB Feeding on Aryl Hydroxylation Activity in the Liver of Rats, Mice, and Hamsters

Each group contained 3—5 animals, and the values are their mean ± SD.
basal diet (○), 0.06% 3'-Me-DAB diet (●)

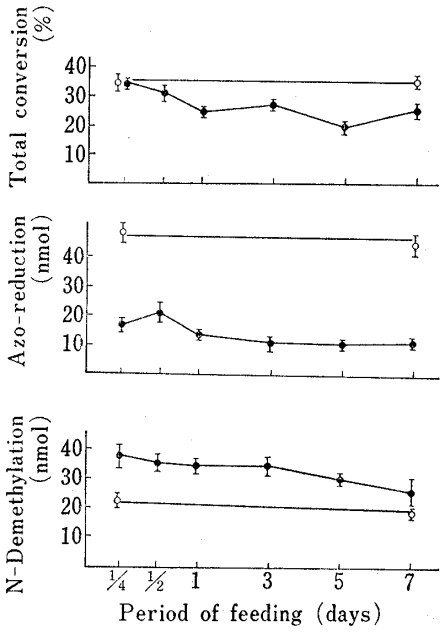

Fig. 7. Effect of 3'-Me-DAB Treatment at the Early Stage of Feeding on the Hepatic Activity for 3'-Me-DAB Metabolism by Rats

Each group contained 3—5 animals, and the values are their mean ± SD.
basal diet (○), 0.06% 3'-Me-DAB (●)

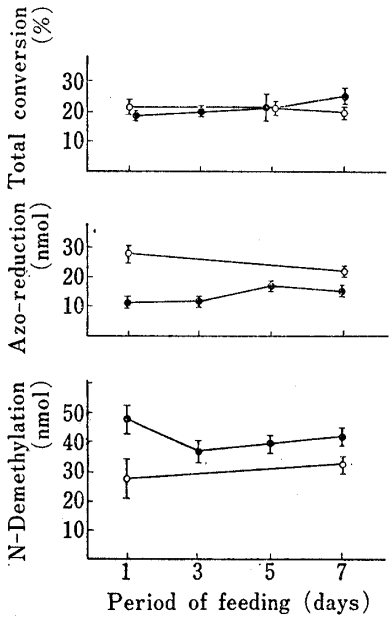

Fig. 8. Effect of 3'-Me-DAB Treatment at the Early Stage of Feeding on the Hepatic Activity for 3'-Me-DAB Metabolism in Mice

Each group contained 3—5 animals, and the values are their mean ± SD.
basal diet (○), 0.06% 3'-Me-DAB diet (●)

in the liver of these animals, aryl hydroxylation was a minor metabolic reaction. The repeated administration of 3′-Me-DAB also did not produce a significant change in the hepatic activity of rats up to the 7th week but, after the 9th week, the activity was gradually depressed in a similar manner as other metabolic reactions.

Effect of 3′-Me-DAB Treatment in Earlier Periods on the Hepatic Activity of Rats and Mice for 3′-Me-DAB Metabolism

The effect of 3′-Me-DAB administration on 3′-Me-DAB metabolism by rat and mouse liver was recognized at early stages of carcinogenesis, especially in the rat liver already at the 1 week of feeding, as shown in Figs. 3, 4, and 5. Therefore, examinations were made to see when the effect of treatment would be observed after first administration of 3′-Me-DAB.

When rats or mice were fed with the 3′-Me-DAB diet for 6 hr or 1 day to 5 days, the amount of 3′-Me-DAB administrated totalled 2.6 mg/rat at 6 hr, 6.8 mg/rat at 12 hr, and the same dose/animal in 1 to 5 days, as shown in Fig. 2. Fig. 7 shows that there was no difference in the total conversion of 3′-Me-DAB to metabolites between the two groups of rats until 12 hr of feeding, but, after 1 day of feeding, the total conversion in the dye-fed group was approximately 10% lower than that of the control. Azo-reduction of 3′-Me-DAB to 3-toluidine in the rat liver was significantly depressed already by 6 hr of feeding and, conversely N-demethylation of 3′-Me-DAB to 3′-Me-MAB was apparently accelerated. In the mouse liver, the total conversion was not affected by their treatment for 1 to 5 days, as shown in Fig. 8, but the azo-reduction activity was depressed and N-demethylation to 3′-Me-MAB was promoted already by 1 day of feeding. It was surprising that such a low dose of 3′-Me-DAB had a great effect on these metabolic activities in the rat and mouse liver.

Discussion

The primary metabolism of 3′-Me-DAB in the liver of rats, mice, and hamsters was the azo-reduction, N-demethylation, and aryl hydroxylation throughout the observation period. N-Demethylation of DAB to secondary amines, N-hydroxylation, and esterification have been demonstrated as the most important metabolism related to the carcinogenic mechanism of the aminoazo dyes, and the azo-reduction, aryl hydroxylation, and N-demethylation to primary amines as the detoxicative metabolism.[4,15] In the liver of control rats, the reductive cleavage of azo linkage was the main pathway for the metabolism of 3′-Me-DAB rather than N-demethylation, while in the liver of mice and hamsters, the latter reaction was the main pathway rather than the former under these experimental conditions, as shown in Fig. 4, 5, and 6.

The effect of 3′-Me-DAB administration on the overall activities of 3′-Me-DAB metabolism by the liver homogenate is shown in Fig. 3, 7, and 8, and there was a distinct correlation between the activity and animal species, the higher the sensitivity of animals to hepatocarcinogenesis by 3′-Me-DAB, the earlier and the greater the suppression of the hepatic activity. By the repeated administration of 3′-Me-DAB to hamsters, in which 3′-Me-DAB is not carcinogenic, the hepatic azo-reduction activity, amount of 3-acetamidotoluene formed, was reduced and the N-demethylation activity, amount of 3′-Me-MAB and 3′-Me-AB formed, was conversely accelerated throughout the experimental period (Fig. 4 and 5). Consequently, the total conversion of 3′-Me-DAB to metabolites in the liver of hamsters fed with the dye was approximately the same as that of the control (Fig. 3).

The azo-reduction activity, amount of 3-toluidine formed, in the liver of rats, in which 3′-Me-DAB is carcinogenic, decreased markedly by 3′-Me-DAB administrations already

15) J.A. Miller and E.C. Miller, *J. Exp. Med.*, **87**, 139 (1948); M. Matsumoto and H. Terayama, *Gann*, **56**, 169 (1965).

from 6 hr later throughout the experimental period (Fig. 4 and 7), and suppression of total conversion in the rat liver by feeding of 3′-Me-DAB diet is mainly due to this decrease. Yamane, *et al.*[9] recently reported a correlation between the effect of some metals on hepatocarcinogenesis by 3′-Me-DAB and azo-reduction activity in the rat liver, the higher the elevation of azo dye reduction activity in the metabolism of amino-azo dyes, the greater the suppression of carcinogenesis. Accordingly, it was suggested that the notable decrease of the azo-reduction activity for 3′-Me-DAB in the rat liver by 3′-Me-DAB administration may be partly responsible for hepatocarcinogenesis by 3′-Me-DAB. N-Demethylation of 3′-Me-DAB to 3′-Me-MAB in the liver of rats and mice was enhanced by their treatment with the dye only at an early period (Fig. 5, 7, and 8). It was of interest that in these experimental periods, from 1st to 3rd week, amount of the protein-bound dye in the rat liver, which was closely related to hepatocarcinogenesis by the amino-azo dyes and detected only in the liver of rats and mice, rose to a maximum peak in the case of 3′-Me-DAB administration.[7,16] It was also reported that the formation of this polar dye[16,17] and the metabolism of aminoazo dyes[10,11,13] were very low in the tumorous liver of rats. In the present work with rats all the metabolic activities were also notably decreased after the 9th week when tumor begun to be observed in the liver, but in the 17th week the metabolic activity in the liver tissues freed from tumor was higher than that in the tumorous liver and corresponded to the results in the 9th week.

Thus, the results obtained with the rats indicate that the metabolism of 3′-Me-DAB by the liver homogenate changed significantly by 3′-Me-DAB administration and this is different from that reported by Takamiya, *et al.*[8] that DAB administrations had no marked influence on DAB metabolizing enzymes throughout the precancerous period. However, the present data also demonstrate that no significant effect of feeding the dye for 6 hr to 7 weeks was recognized on the aryl hydroxylation activity for 3′-Me-DAB (Fig. 6). This observation is in agreement with the previous work by Yamane, *et al.*[9] in which the aryl hydroxylation activity for DAB was measured by colorimetry after feeding of 0.09% DAB diet for 1 to 21 weeks to female Wistar rats.

Determination of N-hydroxylation activity of 3′-Me-MAB, a more proximate carcinogen,[4] is now under investigation in our laboratory.

16) E.C. Miller, J.A. Miller, R.W. Sapp, and G.W. Weber, *Cancer Res.*, **9**, 336 (1949); H. Terayama and M. Matsumoto, *Gann*, **53**, 293 (1962).
17) E.C. Miller and J.A. Miller, *Canser Res.*, **7**, 468 (1947).