EFFECT OF BARBITAL ON CARCINOGENIC ACTION AND METABOLISM OF 4-DIMETHYLAMINOAZOBENZENE*1

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Synopsis

To study the influence of activation of metabolic function of carcinogen to chemical carcinogenesis, effect of barbital, an inducer of enzymes related to drug metabolism, was studied on the development of liver cancer in the rat fed with 4-dimethylaminoazobenzene (DAB), and compared with that of 3-methylcholanthrene,*4 known to antagonize the carcinogenic effect of DAB. In an animal experiment of a long period, the incidence of cancer development was much lower in barbital group than that in 3-methylcholanthrene group. Comparative studies were also made on the quantitative measurement of DAB metabolites in urine and bile, and on the activities of some enzymes in the liver of rats fed with DAB plus barbital diet, DAB diet, or basic diet alone. Excretion of DAB metabolites, especially azo dye glucuronides, in the bile increased in the middle stage of the barbital feeding.

INTRODUCTION

Richardson and Miller9,14) reported the retarding effect of carcinogenic hydrocarbons, such as 3-methylcholanthrene,*4 3,4-benzopyrene, dibenz[a,h]anthracene, or benz[a]anthracene, on DAB carcinogenesis in rat when it was given simultaneously with DAB. Miller9) found that the depression of liver N-demethylase and azo reductase activities by 3'-methyl-DAB was abolished by 3-methylcholanthrene and suggested that the activation of DAB metabolism by 3-methylcholanthrene might be the cause of retardation of carcinogenesis. However, quantitative analysis of DAB metabolites has not been carried out.

*1 A part of this work was reported at the 8th International Cancer Congress, Moscow, 1962, and at the Symposium on the mechanism of chemical carcinogenesis at the 23rd Annual Meeting of the Japanese Cancer Association, Tokyo, 1964.

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*4 The nomenclature of methylcholanthrenes has been changed from 20-methyl- to 3-methylcholanthrene in accordance with IUPAC nomenclature rule (A-23.1). The numbering and structure orientation of cholantherne are given below:

\[ \text{H}_2\text{C} - \text{CH}_2 \]


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These carcinogenic hydrocarbons have been found to be inducers of some enzymes, such as liver N-demethylase,\textsuperscript{2)} ring hydroxylase,\textsuperscript{3,4)} and enzymes related to ascorbic acid cycle.\textsuperscript{1)} Many drugs, such as barbital, aminopyrine, or chloretone, are known as powerful inducers of these enzymes. The work presented here is to investigate the effect of non-carcinogenic metabolic inducer, barbital, on the incidence of liver tumor induced

Fig. 1. Main metabolites of DAB in rat
by DAB, that may provide some information on the relationship between the carcino-
genic activity and metabolic function.

In Expt. I of the present paper, the results of a long-term experiment on the retarding
effect of barbital and 3–methylcholanthrene on DAB carcinogenesis were compared
with each other. Barbital showed a more marked retarding effect than 3–methylcholanth-
rene in the experiment.

In Expt. II, DAB metabolites in urine and bile, and activities of enzymes in the liver
related to DAB metabolism were studied in those animals which had been fed with
barbital plus DAB diet, DAB plus 3–methylcholanthrene diet, or basal diet alone, and
compared with each other.

The main metabolites of DAB which have been identified\textsuperscript{5,6,8,10,15} in urine and
bile of rat are shown in Fig. 1. It has been reported in the preceding paper\textsuperscript{6} that
metabolites retaining the azo linkage are mainly excreted in bile, while those derived
by azo-bond cleavage are excreted in urine.

In this experiment, therefore, the main metabolite in urine, PAP-OS,\textsuperscript{*5} and 11 azo
compounds (DAB, MAB, AB, 4'-OG-DAB, 4'-OG-MAB, 4'-OG-AB, 4'-OG-AB-
NAc, 4'-OS-DAB, 4'-OS-MAB, 4'-OS-AB, and 4'-OS-AB-NAc) in bile were
separately determined by means of thin-layer chromatography followed by colorimetry.

The presence of DAB amine-N-oxide, 3-OG-AB, and 3-OS-AB, which are supposed
to be proximate carcinogens in the DAB metabolites, were also examined, but with
negative results. N-Demethylase and azo reductase activities in liver homogenates were
estimated by the method described by Mueller and Miller.\textsuperscript{12,13}

**Experiment I** (carried out in Sasaki Institute in 1962).

**MATERIALS**

**Animals** Seven-week-old Donryu male rats, supplied by the Central Laboratory for
Experimental Animals, Tokyo, were used at the initial body weight of 70~80 g. They
were housed 4 to a cage, and were given diet and water \textit{ad libitum}.

**Diets** Basal diet: Semisynthetic cube diet, CE2,\textsuperscript{*6} was obtained from the Central
Laboratory for Experimental Animals, Tokyo.

DAB diet: DAB was dissolved in olive oil and this was added to the powder of basal
diet in a concentration of 0.06% and made into a cube diet of the usual size.

DAB+MC diet: DAB and 3–methylcholanthrene were added to the basic diet in a
concentration of 0.06% and 0.0067%, respectively, and made into cubic form in the
same manner as the DAB diet.

DAB+BA diet: This diet contained 0.06% of DAB and 0.06% of barbital.

**METHOD**

Rats were divided into 5 groups, 30 to each, and fed with the following diet for 5
months. Then the feeding was continued on the basal diet for all the groups.

\textsuperscript{*5} For abbreviations, see Fig. 1.

\textsuperscript{*6} Composition of the basal diet: Crude protein 24.3\%, crude fat 4.4\%, carbohydrate 50.1\%,
vegetable fiber 3.8\%, salt mixture 6.9\%, and water 8\%.

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Group I: DAB diet
Group II: DAB+MC diet on Monday and Tuesday, and DAB diet on other days of the week.
Group III: DAB+MC diet
Group IV: DAB+BA diet on Monday and Tuesday, and DAB diet on other days of the week.
Group V: DAB+BA diet

The diet was given three times a week, and the amount was restricted to 14 g/rat/day, which was checked to be consumed entirely by the rats in any of the experimental groups. Each rat was inspected and palpated once a week, for the development of liver cancer. When tumor rats fell into agonized condition, they were sacrificed and submitted to autopsy. On 365th experimental day, all the survivors were sacrificed and submitted to autopsy.

**Histological Examination** The liver, spleen, pancreas, mesentrial and mediastinal lymph nodes, thymus, and upper lobe of the left lung were examined. They were fixed in Bouin's solution and made into paraffin sections. The staining was made with Hematoxylin and Eosin.

**Results**

**Incidence of Liver Cancer**

In Group I, 15 rats died before finishing the months of DAB-feeding. In 13 out of the remaining 15 rats, liver cancer developed by the end of the 8th month. The remaining 2 died of pneumonia in the 6th and 7th month, respectively.

In Group II, only 3 rats died before the end of feeding. By the end of the 11th month, liver cancer developed in 25 out of 27 rats. The remaining 2 died of pneumonia in the 6th month.

In Group III, 5 rats were lost during the feeding. In 18 out of 19 rats, there was a development of liver cancer by the end of 12th month. The other 6 rats died without any tumor between the 6th and 8th month.

In Group IV, 4 rats were lost during the feeding. In 15 out of 17 rats, liver cancer developed by the end of the experiment. Other 9 rats died of pneumonia between the 6th and 11th month.

In Group V, 6 rats died before the 5th month. In 4 out of 6 rats, there were cancers in the liver. However, as many as 18 rats died without any development of tumor between the 6th and 12th month.

The total number of rats which died of tumor, the number of valid animals, and percentage of incidence of tumor by the end of every month from the 6th to 12th month are tabulated in Table I and Fig. 2.

The incidence of liver cancer was much lower in the barbital group than in the control or 3-methylcholanthrene group, and also it was the lowest in Group V in which the animals were fed with DAB+BA diet continuously.
Histological classification of the induced liver cancer is shown in Table II. The incidence of typical hepatoma increased in the order of I, II, III, IV, V and that of parenchymatous hepatoma, in the order of V, IV, III, II, I. The incidence of proliferative change in liver of animals which did not suffer from cancer is shown in Table III.

Table I. Incidence of Liver Cancer

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</table>

Experimental month

No. of tumor animals/No. of animals used. Percentage in parentheses.

Histological Picture

Histological classification of the induced liver cancer is shown in Table II. The incidence of typical hepatoma increased in the order of I, II, III, IV, V and that of parenchymatous hepatoma, in the order of V, IV, III, II, I. The incidence of proliferative change in liver of animals which did not suffer from cancer is shown in Table III.
Metastasis into the Lung

The incidence of metastasis into the upper lobe of the left lung is shown in Table IV. As shown, the incidence was lower in barbital group than in control or 3-methylcholanthene group.

Experiment II (carried out at the Tokyo Biochemical Institute in 1964-1965.)

Materials

Animals Eight-week-old Donryu male rats weighing 170〜190 g were employed. They were bred in an air-conditioned room (temperature 25°±0.5°, relative humidity 55%) and housed 5 rats to a cage.

Diet Basal diet, DAB diet, and DAB+BA diet described in Experiment I were used.

Reagents DAB (commercially available) was purified on alumina column, with benzene as the solvent, and recrystallized from ethanol. m.p. 117°. Other DAB metabolites were synthesized previously by Dr. Y. Hashimoto.
**Method**

Method for Determination of DAB Metabolites

By the method described in a previous paper, an emulsion was prepared from equal volumes of olive oil solution of DAB and rat bile with asciator. One-half ml of the emulsion was injected directly into duodenum under laparatomy, and bile was collected through a polyethylene tubing on which the rat repellent, cycloheximide, was painted. Azo compounds in bile were adsorbed on DEAE-cellulose, eluted with NH₄Cl, hydrolysed by β-glucuronidase and Takadiastase, extracted with ether, and separated by silica gel thin-layer chromatography. Each metabolite was extracted with methanol and determined by spectrophotometry.

PAP-OS in urine was separated by a column of ion-exchange resin (Dowex 1 XI-Cl), hydrolysed by NH₄Cl, and determined by the Indophenol reaction.

**Estimation of Liver Enzyme Activities**

The rats were killed by decapitation and 10% homogenate in 0.75% KCl was prepared from V lobe of the liver. For estimation of enzyme activities, the methods described by Mueller and Miller was modified and used.

(1) N-Demethylase*: A mixture of 0.5 ml of 0.1M Na₂HPO₄-KH₂PO₄ buffer (pH 7.4), 0.2 ml of 0.6M nicotinamide, 0.3 ml of 0.03M glucose 6-phosphate, 0.1 ml of 0.1% NAD, 0.1 ml of 0.1% NADP, 0.1 ml of 0.03M KCN, 0.1 ml of the homogenate, 0.6 ml of 0.75% KCl, and 0.1 ml of 0.1M MgCl₂ was added with 0.1 ml of 0.08% ethanol solution of 3-methyl-MAB and the mixture was incubated at 37°C for 45 mins. with mechanical shaking.

When the incubation was completed, 3 ml of acetone and 4 ml of benzene were added. After shaking and centrifugal separation, the upper layer was evaporated to dryness. To the residue was added 0.5 ml of ether solution of 2,2'-dimethyl-DAB (40 μg/ml) as the internal standard. The sample was developed on silica gel thin layer with benzene:petroleum benzine (2:1). Yellow bands of 2,2'-dimethyl-DAB (Rf 0.91) and 3-methyl-MAB (Rf 0.42) were extracted with methanol, and the dyes were determined spectrophotometrically at 414 and 384 mμ. The amount of 3-methyl-MAB in the sample was calculated by proportion to the internal standard.

(2) Azo reductase: To a mixture of 0.5 ml of 0.1M Na₂HPO₄-KH₂PO₄ buffer (pH 7.4), 0.2 ml of 0.6M nicotinamide, 0.3 ml of 0.03M glucose 6-phosphate, 0.1 ml of 0.1% NAD, 0.1 ml of 0.1% NADP, 1.0 ml of the homogenate, 0.7 ml of 0.75% KCl, and 0.1 ml of 0.1M MgCl₂, 0.1 ml of 0.03% ethanol solution of DAB was added and the mixture was incubated at 37°C for 45 mins. with mechanical shaking. Then 3 ml of 20% CCl₃COOH in acetone:ethanol (1:1) solution was added. After centrifugation, DAB in the supernatant was determined at 525 mμ. Azo reductase activity was shown by the decrease of DAB.

* To avoid a side reaction (azo-bond cleavage, etc.) KCN was added (referred to the work of Kuriyama and Terayama). ATP, which was used in their original work, was not added because it did not seem to influence the results.
Treatments of the Animals

Rats were divided into 3 groups and fed with the following diet for 154 days.

- Group A: DAB diet
- Group B: DAB + BA diet
- Group C: Basal diet

To each cage containing 5 animals, 450 g of the diet was given twice a week and consumption of the diet was estimated.

Before the determination, rats in Groups A and B were fed with basal diet alone for 2 days in order to avoid the influence of the feeding on DAB metabolites, and 11 mg/rat/day of barbital sodium was administered to rats of B group. Then the rats were operated and 5 mg/rat of DAB was injected intraduodenally. Bile and urine were collected for the following 24 hrs. and analysed.

RESULTS

DAB metabolites were determined on the 24th, 45th, 87th, and 157th day of the experiment in 3 cases in each group (Table V and Figs. 3~6). In Group A, excretion of AB increased in the early stage and then decreased. Excretion of 4'-OG-DAB and 4'-OS-DAB was larger in Group A than in C. In Group B, biliary excretion of AB did not decrease. O-Glucuronides, especially 4'-OG-MAB, increased markedly once and then decreased to the normal level (Fig. 7). The main metabolites of DAB, PAP-OS in urine, was not affected by the feeding of barbital or DAB.

Liver enzyme activities were determined on the 6th day, in 2 cases in each group, and on the 21st, 42nd, 84th, and 154th days in 4 cases in each group (Fig. 8). N-Demethylase activity did not change in rats fed with DAB diet. It increased temporarily in rats

Fig 3. Metabolites of DAB in rats fed experimental diets for 3 weeks
Fig. 4. Metabolites of DAB in rats fed experimental diet for 6 weeks
Percentage shows molar ratio to administered DAB (5 mg)

Fig 5. Metabolites of DAB in rats fed experimental diet for 12 weeks

A group
B group
C group
Fig. 6. Metabolites of DAB in rats fed experimental diet for 22 weeks

Fig. 7. Biliary excretion of aminoazobenzene (AB), O-glucuronides (OG), and O-sulfates (OS)

- A group
- B group
- C group

Fig. 7. Biliary excretion of aminoazobenzene (AB), O-glucuronides (OG), and O-sulfates (OS)
fed with DAB+BA diet. Azo reductase decreased in rats fed with DAB diet and in rats fed with DAB+BA diet to the same extent.

Liver enzyme activities were also determined in rats used for the determination of metabolites (Fig. 9). Azo reductase decreased in control rats as well as in rats fed with DAB diet.

To examine the influence of interruption of DAB feeding, rats in A or B group were fed with basal diet for 2 or 6 days after 42nd, 84th, or 154th day of the experiment. To animals in B group, 11 mg/rat/day of barbital sodium was administered during the interruption of DAB feeding. Then, enzyme activities in livers of the rats were determined (Fig. 10).

None of the metabolites was found in bile at 24 hours after the interruption of DAB feeding. A small amount of PAP-OS was found in urine still on 3rd day after the interruption of DAB feeding.
AB-NAc, 3-OG-AB, 3-OS-AB, and DAB amine-N-oxide in bile and PAP-OG in urine could not be detected (the sensitivity of the present method: 5 μg of AB-NAc, 3-OG-AB, 3-OS-AB, and DAB amine-N-oxide, and 20~30 μg of PAP-OG per sample).

Consumption of DAB is shown in Table VI. Significant difference could not be found between A and B groups.

After completion of the feeding, 7 rats of each group were fed with basal diet, and when the rats died, they were submitted to autopsy. At 340th day, all the survivors were sacrificed and submitted to autopsy. The results are shown in Table VII. The period for incidence of liver cancer in rats of A and B groups was later than that in rats of I and V groups in Experiment I. This might be due to the environmental difference of two experiments.

Fig. 10. Effect of interruption of DAB feeding on enzyme activities after long feeding

AB-NAc, 3-OG-AB, 3-OS-AB, and DAB amine-N-oxide in bile and PAP-OG in urine could not be detected (the sensitivity of the present method: 5 μg of AB-NAc, 3-OG-AB, 3-OS-AB, and DAB amine-N-oxide, and 20~30 μg of PAP-OG per sample).

Consumption of DAB is shown in Table VI. Significant difference could not be found between A and B groups.

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<th>AB (%)</th>
<th>4'-OG-DAB (%)</th>
<th>4'-OG-MAB (%)</th>
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<td>36.0</td>
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<td>4</td>
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<td>0.20</td>
<td>3.54</td>
<td>0.45</td>
<td>1.01</td>
<td>0.22</td>
<td>1.17</td>
<td>0.42</td>
<td>0.50</td>
<td>1.10</td>
<td>0.90</td>
<td>9.60</td>
<td>42.0</td>
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</tr>
<tr>
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<td>2</td>
<td>0.15</td>
<td>0.15</td>
<td>4.86</td>
<td>0.39</td>
<td>0.86</td>
<td>0.22</td>
<td>0.91</td>
<td>0.97</td>
<td>0.92</td>
<td>1.75</td>
<td>1.61</td>
<td>12.74</td>
<td>43.5</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>0.18</td>
<td>0.24</td>
<td>2.45</td>
<td>0.12</td>
<td>0.22</td>
<td>0.24</td>
<td>1.04</td>
<td>0.56</td>
<td>0.68</td>
<td>1.56</td>
<td>1.77</td>
<td>8.90</td>
<td>57.8</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>0.13</td>
<td>0.37</td>
<td>2.62</td>
<td>0.18</td>
<td>0.37</td>
<td>0.11</td>
<td>0.88</td>
<td>0.32</td>
<td>0.51</td>
<td>1.13</td>
<td>1.54</td>
<td>7.79</td>
<td>49.5</td>
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<tr>
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<td>0.08</td>
<td>2.42</td>
<td>0.15</td>
<td>0.38</td>
<td>0.18</td>
<td>1.00</td>
<td>0.60</td>
<td>0.46</td>
<td>1.45</td>
<td>1.44</td>
<td>8.22</td>
<td>51.3</td>
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</tbody>
</table>

Percentage shows molar ratio of metabolites to administered DAB (5 mg).
DISCUSSION

Metabolic sequence of a carcinogen in the body should be an important factor to influence the induction of cancer. Richardson and Miller observed a retarding effect of carcinogenic hydrocarbons, which have enzyme-inducing effect, on DAB carcinogenesis of rats, and suggested a close relationship between the metabolism of carcinogen and its carcinogenicity. These hydrocarbons also showed a retarding effect on the development of liver carcinoma by 2-acetamidofluorene in rats.11)

These hydrocarbons are known as inducers of some enzymes, such as N-demethylase, ring hydroxylase, and related enzymes of ascorbic acid cycle. Many other substances, barbiturates, some pyrazolone derivatives, and anabolic steroids are known to have similar effect of enzyme activation. In Experiment I of this paper, retarding effect of barbital on DAB carcinogenesis was shown and it was indicated that an enzyme inducer other than carcinogenic hydrocarbons also has a retarding effect on DAB carcinogenesis.

In Experiment II, urinary and biliary metabolites of DAB in rats fed with DAB and DAB plus barbital for a long period were determined by the technique described in a previous paper. The data obtained indicate that the excretion of hydroxylated metabolites in bile was significantly promoted by barbital. N-Demethylase activity did not change in rats fed with DAB, which was different from the results of Miller and of Matsumoto et al. Azo reductase activity was decreased by DAB feeding and the

Table VI. Consumption of DAB (mg/rat/day)

<table>
<thead>
<tr>
<th>Week</th>
<th>1st~5th</th>
<th>6th~10th</th>
<th>11th~15th</th>
<th>16th~22nd</th>
</tr>
</thead>
<tbody>
<tr>
<td>A group</td>
<td>10.2</td>
<td>10.9</td>
<td>11.0</td>
<td>10.0</td>
</tr>
<tr>
<td>B group</td>
<td>10.0</td>
<td>10.8</td>
<td>11.5</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Table VII. Histological Aspect of Liver of Rats at the End of Feeding

<table>
<thead>
<tr>
<th>Group</th>
<th>Slight proliferation</th>
<th>Adenoma</th>
<th>Cancer(^a)</th>
<th>Days of feeding until death(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>++</td>
<td>++</td>
<td>B-b fibrosarcoma behind II lobe of liver</td>
<td>164</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>213</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>226 pneumonia</td>
</tr>
<tr>
<td>A 4</td>
<td>++</td>
<td>++</td>
<td>A-a</td>
<td>338</td>
</tr>
<tr>
<td>B 4</td>
<td>+</td>
<td>+</td>
<td>B-b</td>
<td>328</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>287 pneumonia</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>305 pneumonia</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>314 leukemia</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>*</td>
</tr>
</tbody>
</table>

\(a\) Type of cancer is shown by the same expression as used in Table II.

\(b\) The rats marked with * were sacrificed on the 340th day of the experiment.
simultaneous feeding of barbital did not influence this effect. Even a single administration of DAB decreased azo reductase activity. On the other hand, by interruption of DAB feeding, azo reductase activity recovered within a week. Therefore, depression of azo reductase activity by DAB seemed to be an acute and reversible change. Of the metabolites detected so far, no carcinogenic intermediate was found except MAB.

From these experiments, the retarding effect of barbital on the incidence of liver cancer by DAB in a rat might be closely related to the acceleration of metabolism of the carcinogen.

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13) Idem, ibid., 180, 1125 (1949).