The N-Hydroxy Metabolites of N-Methyl-4-aminoazobenzene and Related Dyes as Proximate Carcinogens in the Rat and Mouse

Elizabeth C. Miller, Fred F. Kadlubar, James A. Miller, Henry C. Pitot, and Norman R. Drinkwater

ABSTRACT

The carcinogenicities for rats and mice of N-methyl-4-aminoazobenzene (MAB) and its hepatic microsomal metabolite N-hydroxy-N-methyl-4-aminoazobenzene (N-hydroxy-MAB) were compared under several conditions. N-Ethyl-4-aminoazobenzene, 4-aminoazobenzene, and their N-hydroxy derivatives were also included in some of the assays.

About 25% of the rats given MAB or N-hydroxy-MAB (3 to 5 mmol/kg body weight) by stomach tube over a 5-week period developed hepatic tumors by 18 to 22 months. Similarly treated rats subsequently given phenobarbital in the drinking water until the termination of the experiment developed about twice as many hepatic tumors. N-Hydroxy-MAB, administered p.o., but not MAB, also induced multiple papillomas and extensive carcinomas of the forestomach in approximately 50% of the rats. Only low incidences of hepatocellular carcinomas occurred in partially hepatectomized rats given a single i.p. injection of 180 μmol/kg body weight of MAB or N-hydroxy-MAB with or without subsequent administration of phenobarbital. Although repeated s.c. doses of N-benzoyloxy-N-methyl-4-aminoazobenzene induced sarcomas at the site of injection in 90% of the rats, only 3 of 20 rats developed sarcomas at the site of s.c. injections of N-hydroxy-MAB. N-Ethyl-4-aminoazobenzene, 4-aminoazobenzene, and their N-hydroxy derivatives did not induce significant numbers of tumors in any of the above assay systems.

Administration to preweanling male mice of MAB, N-hydroxy-MAB, N-hydroxy-N-ethyl-4-aminoazobenzene, and N-hydroxy-4-aminoazobenzene resulted in high incidences and high multiplicities of hepatic tumors (averages of 5 to 7 tumors/mouse) within 1 year. N-Ethyl-4-aminoazobenzene and 4-aminoazobenzene also induced hepatic tumors under the same conditions, but they were less active.

These data support the conclusion that the N-hydroxy metabolites of these aminoazo dyes are proximate carcinogens.

INTRODUCTION

MAB is oxidized to N-hydroxy-MAB by rat hepatic microsomes in a NADPH-dependent, cytochrome P-450-independent reaction that is catalyzed by a flavoprotein mixed-function oxidase (17). The N-hydroxy metabolite is a substrate for a 3′-phosphoadenosine 5′-phosphosulfate-dependent sulfotransferase in rat liver cytosol (18). The resulting product, presumably MAB-N-sulfate, is a strong electrophile that reacts readily with cellular nucleophiles (18). The major product of the in vitro reaction of MAB-N-sulfate or of the analogous synthetic ester N-benzoyloxy-MAB with guanosine is N-(guanosin-8-yl)-N-methyl-4-aminoazobenzene, and the same adduct is the major derivative obtained on degradation of the hepatic DNA and rRNA from the livers of rats given MAB (22, 23). These data, as well as the enhancement of the carcinogenicity of the structurally related dye 3′-methyl-N,N-dimethyl-4-aminoazobenzene by dietary supplementation with sodium sulfate (3), suggest that MAB-N-sulfate is an ultimate carcinogenic metabolite of MAB in rat liver. In earlier work, a similar sequence of metabolic events for 2-acetylaminofluorene (30), the carcinogenicity and electrophilic reactivity of esters of N-hydroxy-2-acetylaminofluorene (2, 30, 33), and the enhancement of the carcinogenicity of N-hydroxy-2-acetylaminofluorene under special conditions by dietary sodium sulfate (45) similarly implicated N-hydroxy-2-acetylaminofluorene and its sulfuric acid ester as major proximate and ultimate carcinogens, respectively, of 2-acetylaminofluorene in male rat liver in vivo. In accordance with its role as a proximate carcinogenic metabolite, N-hydroxy-2-acetylaminofluorene is a more potent carcinogen in a wider variety of tissues than is the parent amide in rats and other rodents (27, 28).

Although N-hydroxy-MAB is readily oxidized by molecular oxygen (17) and probably has a very short half-life in vivo, several experiments were carried out to determine its carcinogenic activity in rats and mice as a measure of its activity as a proximate carcinogenic metabolite of MAB. In some of these experiments, phenobarbital was administered to rats subsequent to the dyes to promote hepatic tumor formation. Promotion of hepatic tumor induction by long-term administration of phenobarbital has been demonstrated in rats fed 2-acetylaminofluorene (36, 37) or 2-methyl- 3′-methyl-N,N-dimethyl-4-aminoazobenzene (19, 20). The carcinogenicities of EAB, AB, and their N-hydroxy metabolites (17, 41) were also studied in some experiments. EAB (and its N,N-diethyl analog) and AB have shown little or no carcinogenic activity on long-term administration in the diets of adult rats (1, 31, 42). The structures of the dyes investigated in this study are shown in Chart 1.

MATERIALS AND METHODS

Chemicals

MAB (m.p. 89°) (31), EAB (m.p. 87°) (31), and N-benzoyloxy-MAB (m.p. 93°) (39, 46) were synthesized by published methods. 2-Aminoazulene (m.p. 94°) was prepared (35) from the diethyl ester of 2-aminoazulene-1,3-dicarboxylic acid...
Animal Experiments

Male random-bred CD rats and male Fischer rats were obtained from the Charles River Breeding Laboratory (Wilmington, Mass.). The mice were bred in our laboratory from stock random-bred CD-1 mice obtained from the same commercial source. The rats were caged singly in screen-bottomed cages, while the mice were housed in groups of 4 or 5 in plastic cages on hardwood chips (Betta-Chip; Northeastern Products Corp., Warrensburg, N. Y.). Except where noted, all of the rats and mice were fed Wayne Breeder Blox (Allied Mills, Inc., Chicago, Ill.) ad libitum. All of the compounds were dissolved in sterile trioxotanoin for administration, and each experiment contained a control group that received the trioxotanoin. Sodium phenobarbital (0.05 or 0.1%; U.S.P.; Mallinckrodt, Inc., St. Louis, Mo.) was included in the drinking water for some of the rats; fresh solutions were furnished 3 times weekly. All the animals were weighed at least monthly. The rats that received compounds by stomach tube were weighed weekly during the period of dye administration; each litter of preweaning mice was weighed prior to each injection.

Administration p.o. to Rats. Male random-bred CD rats (21 days old) were fed the following purified diet: vitamin-free casein (Teklad Test Diets, Madison, Wis.), 180 g; corn oil (Mazola; CPC International, Inc., Englewood Cliffs, N. J.), 50 g; Phillips and Hart salt mixture (Teklad Test Diets), 40 g; glucose monohydrate (Cerelose; CPC International, Inc.), 727 g; choline citrate, 3 g; vitamin A acetate, 15,000 units; vitamin D₃, 44 μg; α-tocopherol, 48 mg; calcium pantothenate, 7 mg; thiamine hydrochloride, 3 mg; pyridoxine hydrochloride, 2.5 mg; riboflavin, 2 mg; and 2-methyl-1,4-naphthoquinone, 20 mg. This diet, which contains a relatively low level of riboflavin, facilitates the induction of liver tumors on p.o. administration of MAB or N,N-dimethyl-4-aminoazobenzene (29, 32). One week later, the rats were divided into groups of 20, and each rat was given 200 μmol of MAB, N-hydroxy-MAB, EAB, N-hydroxy-EAB, N-hydroxy-AB, or 2-aminoazulene per kg body weight per 2.5 ml trioxotanoin by stomach tube 3 or 5 times per week for 5 to 12 weeks as indicated in Table 1. Each group of rats was transferred to Wayne Breeder Blox pellets 1 week after dye administration was completed for that group. All of the rats in Experiment 1 were laparotomized at 12 months for assessment of liver tumor incidences. The rats of Experiments 1 and 2 were killed for autopsy at 18 and 22 months, respectively.

Injection i.p. into Partially Hepatectomized Rats. Male random-bred CD rats with initial weights of 120 to 140 g were subjected to a two-thirds hepatectomy (12) and 22 to 24 hr later were given injections of trioxotanoin solutions of MAB, N-hydroxy-MAB, EAB, N-hydroxy-EAB, or N-hydroxy-AB. The dose selected for the first experiment was 200 μmol per kg body weight per ml trioxotanoin, but few of the partially hepatectomized rats that received this level of N-hydroxy-MAB lived beyond 1 week. Accordingly, N-hydroxy-MAB was administered in this experiment at the level of 180 μmol/kg body weight. In the second experiment, each of the compounds was administered at the latter dose. About 30% of the partially hepatectomized rats that received 180 μmol of N-hydroxy-MAB died within 1 week after injection of the dye; the early deaths in the other groups were only 0 to 7%. These experiments were terminated at 22 months.

Injection s.c. into Rats. Male Fischer rats were given s.c.
Incidences of tumors in rats fed aminoazo dyes by stomach tube

Charles River random-bred male CD rats were given, starting at 28 days of age (average weights 60 to 80 g), 200 µmol of compound per kg body weight per 2.5 ml of trioctanoin per dose with the frequencies and for the total periods indicated. Where indicated, administration of 0.1% of sodium phenobarbital in the drinking water was started 1 week after the dye administration was completed. There were 20 rats per group, and Experiments 1 and 2 were terminated at 18 and 22 months, respectively. See “Materials and Methods” for further details. The statistical treatment of these data is given under “Results.”

<table>
<thead>
<tr>
<th>Group</th>
<th>Dye administered</th>
<th>No. of rats</th>
<th>No. of rats with liver tumors by 12 mos.</th>
<th>No. of rats with forestomach tumors by 18 mos.</th>
<th>Other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>12 mos.</td>
<td>18 mos.</td>
<td>22 mos.</td>
</tr>
<tr>
<td>1</td>
<td>N-Hydroxy-MAB</td>
<td>5</td>
<td>5</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>MAB</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>5</td>
<td>5</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>5</td>
<td>8</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>5</td>
<td>8</td>
<td>+</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>5</td>
<td>12</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>7</td>
<td>N-Hydroxy-AB</td>
<td>5</td>
<td>5</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>(Solvent only)</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>5</td>
<td>5</td>
<td>+</td>
<td>18</td>
</tr>
</tbody>
</table>

* The 12-month liver tumor incidences were obtained by examination of the livers after laparotomy.

b Only the rats with gross carcinomas were tabulated under “Carcinomas.” The rats that had papillomas with areas of carcinoma in situ or focal carcinoma were included under “Papillomas.”

c Numbers in parentheses, total number of liver tumors or forestomach papillomas in the group of rats.

d Incidences significantly different (p < 0.05) from those for the appropriate solvent control groups.

e Acinar cell carcinoma of the pancreas.
f One rat developed an epidermoid carcinoma of the ear duct gland, and another had a s.c. fibromyxosarcoma.
g Epidermoid carcinoma of the ear duct gland.
h One rat developed an epidermoid carcinoma of the ear duct gland, and another had a s.c. fibroma.
i One rat each developed a soft tissue osteogenic sarcoma of the stomach wall, a fibrosarcoma in the thoracic cavity, an epidermoid carcinoma of the skin, and a reticulum cell sarcoma in the mesentery lymph nodes.
j One rat each developed a s.c. sarcoma, mammary fibroadenoma, sebaceous gland carcinoma of the ear duct gland, stromal nephroma, and adenocarcinoma of the small intestine.
k One rat each developed a fibrosarcoma of the skin and an epidermoid carcinoma of the ear duct gland.
l Tubular adenoma of the kidney.
m s.c. fibroma.
n Generalized lymphoma.
o One rat each developed an adenocortical carcinoma and a mammary carcinoma.
p One rat each developed a keratoacanthoma of the skin, a s.c. lipofibrosarcoma, and a neurofibroma of the jaw.
q One rat developed lymphatic leukemia and another had a s.c. angiosarcoma.

Injections of 10 µmol of compound in 0.2 ml of trioctanoin twice weekly for 12 weeks in the right hind leg. The injection sites were examined at 2- to 4-week intervals for tumors, and the experiment was terminated at 19 months.

Injection i.p. into Preweanling Mice. Male mice were treated by i.p. injection within 24 hr after birth and on Days 8, 15, and 22 with 10 µl of 0.03 M or 0.04 M solutions of MAB or N-hydroxy-MAB and 0.03 M solutions of EAB, N-hydroxy-EAB, AB, N-hydroxy-AB, or 2-aminooazulene in trioctanoin per g body weight. The mice were weaned at 22 days, and Experiments 1 and 2 were terminated at 11 and 12 months, respectively.

Autopsies. All of the rats and mice were subjected to routine gross autopsies which included examination of the skin, s.c. tissue, mammary and ear duct glands, and the organs of the abdominal and thoracic cavities. The rat livers were sliced at approximately 0.5-cm intervals to facilitate detection of tumors. The numbers of individual gross tumors in the forestomachs of the rats that received the dyes by stomach tube were counted. The gross tumors and other abnormal tissues were fixed in 10% neutral formalin, sectioned at 5 to 6 µm, and stained with hematoxylin and eosin.

Statistical Evaluations. Comparisons of tumor incidence were made by Fisher’s exact test (Ref. 5, p. 195). Differences in tumor multiplicity were examined by the Mann-Whitney (Wi-
RESULTS

Repetitive p.o. Administration to Rats. In preliminary experiments, 28-day-old rats that received MAB or N-hydroxy-MAB, 250 μmol per kg body weight per day, died within 1 week, while administration of 200 μmol per kg per day for 5 days each week permitted survival of all of the rats with near-normal weight gains. Thus, in Experiment 1, the rats in each of the groups gained an average of 88 to 110 g in the first 4 weeks and, except for the rats fed MAB for 12 weeks, their gains averaged 470 to 500 g over the first 4 months. The rats in the latter group gained an average of 385 g over the 4-month period. The weight gains were similar for the rats in Experiment 2, and the survival of each group of rats in both experiments was at least 85% at 12 months.

Larger total doses of MAB administered by stomach tube resulted in higher incidences of hepatic tumors (Table 1, Groups 2, 4, and 6). For rats not given phenobarbital, the increase in incidence was 8% per mmol MAB per kg body weight (91% confidence limits, 5 and 11%). The latent period before tumor development decreased with the increase in number of doses of MAB.

In Experiment 1, N-hydroxy-MAB (total dose of 5 mmol/kg body weight) induced a significantly greater (p < 0.002) incidence of hepatic tumors than did MAB if the i.g. administration of these dyes was followed by continuous treatment with phenobarbital (compare Groups 1 and 3). No significant difference between the abilities of these dyes to induce liver tumors was noted in Experiment 2, in which total doses of 3 mmol/kg were administered.

Continuous administration of phenobarbital after the doses of MAB or N-hydroxy-MAB had been completed resulted in higher incidences of hepatic tumors than were observed in dye-fed rats not given phenobarbital. These differences were statistically significant on comparison of Groups 4 and 5 (p < 0.04) and Groups 12 and 13 (p < 0.07), all of which received MAB, and on comparison of Groups 10 and 11 (p < 0.005), treated with N-hydroxy-MAB.

In Experiment 2, 4 rats fed EAB (Group 16) developed solitary hepatic tumors; the incidence was not increased by subsequent administration of phenobarbital (Group 17). One rat treated only with N-hydroxy-EAB and 3 rats treated with N-hydroxy-EAB and then given phenobarbital developed solitary hepatic tumors (Groups 14 and 15). However, neither of these dyes, nor N-hydroxy-AB, nor the easily oxidized aromatic amine 2-aminoazulene showed statistically significant hepatic carcinogenic activity under the conditions of this experiment.

Most of the hepatic tumors that developed in these rats, regardless of the dye administered, were hepatocellular carcinomas. Some of the carcinomas were mixed hepatocellular and cholangiocellular carcinomas. A few of the tumors were cholangiocellular carcinomas without obvious areas of hepatocellular carcinoma in the histological sections examined.

The carcinogenicity of N-hydroxy-MAB at a site of application was evident from the development of tumors of the forestomach in approximately one-half of these rats given the dye p.o. (p < 0.001) (Groups 1, 10, and 11). One rat in the first experiment and 10 rats in the second experiment had invasive epidermoid carcinomas, which usually occupied much of the forestomach; 6 of these carcinomas metastasized to the mesentery and abdominal organs. The rest of the tumors were papillomas that ranged from 2 to 8 mm in diameter. Some of these papillomas showed focal areas of squamous carcinoma or carcinoma in situ. The incidence of these tumors was not influenced by the administration of phenobarbital and, with the exception of one epidermoid carcinoma (about 1 cm in diameter) in a rat fed N-hydroxy-EAB, tumors in the forestomach were not observed in rats from any of the other groups. The tumors in the forestomach generally occurred in rats that died or were killed 18 to 22 months after initiation of the experiments.

Administration i.p. to Partially Hepatectomized Rats. These experiments were based on studies from several laboratories (6—8, 25), which showed that rats or mice subjected to partial hepatectomies 18 to 24 hr prior to the administration of single large doses of certain carcinogens developed hepatic tumors after long latent periods. In our experiments, rats that had received a two-thirds hepatectomy were given injections of a single dose of one of the dyes 22 to 24 hr later (Table 2). Since the dose selected for Experiment 1 (200 μmol/kg body weight) killed about 70% of the rats given N-hydroxy-MAB within 1 week, this dye was then administered to another group at a level of 180 μmol/kg with a loss of about 25% of the rats. The latter dose was used for all of the groups in Experiment 2. Very few deaths occurred from the administration of 200 (Experiment 1) or 180 (Experiment 2) μmol/kg doses of any of the other compounds to the partially hepatectomized rats. The rats that received the injections of N-hydroxy-MAB gained about 250 g over the first 6 weeks, while the rats in the other groups had average weight gains of about 300 g. The average body weight gains were 525 to 560 g for all groups at 5 months.

A single i.p. dose of 180 μmol of MAB per kg body weight in partially hepatectomized rats appeared to be just sufficient to initiate hepatocellular carcinomas. Thus, 5 of the 30 rats given this dose and then given phenobarbital in their drinking water for the duration of the experiment developed hepatocellular carcinomas by the termination of the experiment at 22 months (Experiment 2). This incidence was marginally significant (p < 0.1) on comparison with the incidence for the rats given phenobarbital but not treated with an aminoazo dye. These tumors were all found in rats killed at 22 months and ranged from 4 mm to 2 cm in diameter. Three of the rats that received N-hydroxy-MAB and subsequent long-term treatment with phenobarbital also developed gross hepatocellular carcinomas. No more than one rat in any other group, including those that received the single injection of MAB or N-hydroxy-MAB without subsequent administration of phenobarbital, developed a gross hepatocellular carcinoma.

Sarcomas associated with the laparotomy scar or in the abdominal cavity occurred in low incidence in many of the groups (Table 2). The incidence of these tumors was marginally significant (p < 0.1) for the rats given injections of N-hydroxy-MAB in Experiment 1, but not for any of the other groups. Occasional renal carcinomas and a variety of other tumors also occurred late in the experiments.

Repetitive s.c. Injections into Rats. The repetitive s.c. injections (total dose of 240 μmol) of N-benzoyloxy-MAB and N-hydroxy-MAB permitted slower weight gains than were ob-
N-Hydroxy-MAB as a Proximate Carcinogen

Table 2
Tumor induction in male rats given a single i.p. injection of an aminoazo dye 22 to 24 hr after partial hepatectomy

Male random-bred CD rats (initial weights of 210 to 240 g) were subjected to a two-thirds hepatectomy and given i.p. injections of 180 or 200 μmol of dye per kg body weight per ml of trioctanoin 22 to 24 hr later. The former dose was given to the rats that received N-hydroxy-MAB in Experiment 1 and to all of the rats in Experiment 2. Some rats in Experiment 2 were given phenobarbital in the drinking water from 1 week after injection of the dye to the termination of the experiment; the water contained 0.05% sodium phenobarbital for the first week and 0.1% thereafter. See “Materials and Methods” for further details. The statistical treatment of these data is presented under “Results.”

<table>
<thead>
<tr>
<th>Compound injected</th>
<th>Phenobarbital</th>
<th>Initial no. of rats</th>
<th>1 mos.</th>
<th>16 mos.</th>
<th>18 mos.</th>
<th>22 mos.</th>
<th>Hepato-cellular carcinomas</th>
<th>Sarcomas near injection site</th>
<th>Renal tumors</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Hydroxy-MAB</td>
<td></td>
<td>57</td>
<td>42</td>
<td>41</td>
<td>34</td>
<td>19</td>
<td>1</td>
<td>6&lt;sup&gt;a&lt;/sup&gt; (5, abdominal cavity; 1, laparotomy scar)</td>
<td>1 carcinoma</td>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>N-Hydroxy-EAB</td>
<td></td>
<td>31</td>
<td>30</td>
<td>27</td>
<td>22</td>
<td>11</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 (laparotomy scar)</td>
<td>0</td>
<td>3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>N-Hydroxy-AB</td>
<td></td>
<td>32</td>
<td>30</td>
<td>25</td>
<td>21</td>
<td>14</td>
<td>0</td>
<td>2 (laparotomy scar)</td>
<td>1 carcinoma</td>
<td>1&lt;sup*e&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAB</td>
<td></td>
<td>28</td>
<td>26</td>
<td>21</td>
<td>16</td>
<td>4</td>
<td>1</td>
<td>2 (laparotomy scar)</td>
<td>0</td>
<td>3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>EAB</td>
<td></td>
<td>30</td>
<td>29</td>
<td>27</td>
<td>21</td>
<td>12</td>
<td>1</td>
<td>3 (2, laparotomy scar)</td>
<td>1 malignant hypernephroma</td>
<td>4&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Trioctanoin only)</td>
<td></td>
<td>27</td>
<td>27</td>
<td>25</td>
<td>21</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td></td>
<td>1 myelolipoma</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Hydroxy-MAB</td>
<td></td>
<td>40</td>
<td>29</td>
<td>28</td>
<td>25</td>
<td>17</td>
<td>1</td>
<td>2 (1, laparotomy scar; 1, abdominal cavity)</td>
<td>1 carcinoma</td>
<td>2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>39</td>
<td>30</td>
<td>25</td>
<td>19</td>
<td>11</td>
<td>3</td>
<td>3 (1, laparotomy scar; 2, abdominal cavity)</td>
<td>0</td>
<td>5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAB</td>
<td></td>
<td>29</td>
<td>29</td>
<td>27</td>
<td>24</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td></td>
<td>1 anaplastic nephroblastoma</td>
</tr>
<tr>
<td>(Trioctanoin only)</td>
<td></td>
<td>28</td>
<td>28</td>
<td>26</td>
<td>18</td>
<td>16</td>
<td>0</td>
<td>1 (laparotomy scar)</td>
<td>0</td>
<td>2&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>28</td>
<td>28</td>
<td>27</td>
<td>23</td>
<td>19</td>
<td>1</td>
<td>1 (laparotomy scar)</td>
<td>0</td>
<td>3&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Incidences significantly greater (p = 0.09) than those for the appropriate solvent control group.
<sup>b</sup> Two rats had squamous cell papillomas of the skin, and one rat had a mammary adenocarcinoma and a malignant hemangiopericytoma of the adrenal.
<sup>c</sup> Another rat had a benign hepatoma.
<sup>d</sup> One rat each developed a malignant lymphoma, epidermoid carcinoma of the ear duct gland, and pheochromocytoma of the adrenal.
<sup>e</sup> s.c. fibroma.
<sup>f</sup> Two rats each developed a s.c. fibroma, and one rat had a sarcoma arising in the penis.
<sup>g</sup> Two rats developed s.c. sarcomas distant from the injection site, one developed a sarcoma in the liver, and one had an adenocortical carcinoma.
<sup>h</sup> Mammary fibroadenoma.
<sup>i</sup> One rat each developed a malignant fibroadenoma, and another rat developed a mesothelioma in the thoracic cavity.
<sup>j</sup> Two rats developed epidermoid carcinomas of the ear duct gland, one had a sarcoma arising in the skin, one had a squamous cell carcinoma of the skin, and one had a mixed transitional cell and squamous carcinoma of the urinary bladder.
<sup>k</sup> Also involved the spleen.
<sup>l</sup> One rat each developed a s.c. fibroma, s.c. neurofibroma, s.c. sarcoma, mammary adenocarcinoma, fibrosarcoma of the ear lobe, and leiomyosarcoma of the small intestine.
<sup>m</sup> Two rats developed epithelial carcinomas of the ear duct gland, and one had a s.c. sarcoma.
<sup>n</sup> One rat each developed a Kupffer cell sarcoma of the liver and a s.c. sarcoma.
<sup>o</sup> One rat each developed a squamous cell carcinoma of the stomach, a squamous cell carcinoma of the skin, and a s.c. sarcoma.

Repeated injections of N-benzoyloxy-MAB induced sarcomas at the injection site in 18 of 20 rats (p < 0.001). The first sarcoma was observed at 5 months, and the last sarcoma-bearing rat was killed at 15 months. This finding is consistent with earlier reports from this laboratory on the induction of sarcomas at the injection site by this ester of N-hydroxy-MAB and N-hydroxy-EAB and N-hydroxy-AB, this dose of N-hydroxy-EAB caused a high mortality by 7 days. Accordingly, the doses of EAB and N-hydroxy-EAB were reduced to 0.3 μmol/g body weight in Experiment 1. All of the dyes were injected at the latter level in Experiment 2.

Male CD-1 mice characteristically develop gross hepatomas (type A) spontaneously starting at about 9 months of age. The overall incidence of tumor-bearing mice by 12 months in the controls for these experiments was 20 to 30%, and the average number of tumors per mouse was 0.2 to 0.6 (Table 4, un.injected controls and mice that received injections of trioctanoin only). Administration of MAB, EAB, AB, or their N-hydroxy derivatives in 4 doses from the first to the 22nd day after birth (total dose of 1.2 μmol/g body weight) increased the percentage of tumor-bearing mice to 80 to 90% and greatly increased the average tumor multiplicity (1.6 to 7.4 hepatomas/mouse). The hepatic tumors in the mice that received each of the dyes were diagnosed primarily as type A hepatomas (15); however, some were classified as type B hepatomas and some as mixed

Injection i.p. of Male CD-1 Mice Prior to Weaning. Although newborn mice tolerated 0.4-μmol/g body weight doses of either MAB or N-hydroxy-MAB, this dose of N-hydroxy-EAB caused a high mortality by 7 days. Accordingly, the doses of EAB and N-hydroxy-EAB were reduced to 0.3 μmol/g body weight in Experiment 1. All of the dyes were injected at the latter level in Experiment 2.

Male CD-1 mice characteristically develop gross hepatomas (type A) spontaneously starting at about 9 months of age. The overall incidence of tumor-bearing mice by 12 months in the controls for these experiments was 20 to 30%, and the average number of tumors per mouse was 0.2 to 0.6 (Table 4, un-injected controls and mice that received injections of trioctanoin only). Administration of MAB, EAB, AB, or their N-hydroxy derivatives in 4 doses from the first to the 22nd day after birth (total dose of 1.2 μmol/g body weight) increased the percentage of tumor-bearing mice to 80 to 90% and greatly increased the average tumor multiplicity (1.6 to 7.4 hepatomas/mouse). The hepatic tumors in the mice that received each of the dyes were diagnosed primarily as type A hepatomas (15); however, some were classified as type B hepatomas and some as mixed.

SEPTEMBER 1979

3415

Downloaded from cancerres.aacrjournals.org on May 1, 2017. © 1979 American Association for Cancer Research.
type A-type B hepatomas. The biological behavior of both the type A and type B hepatomas and their designations as malignant neoplasms have been considered in an earlier publication (10).

In both experiments, the average number of liver tumors per mouse was similar for the mice that received MAB or N-hydroxy-MAB. However, both N-hydroxy-EAB (in both experiments) and N-hydroxy-AB (administered only in Experiment 2) induced substantially more tumors than did their parent dyes (p < 0.001 and < 0.01, respectively). The ratios of the average number of tumors per liver for mice that received N-hydroxy-EAB as compared to EAB in Experiments 1 and 2 were 3.9 and 1.9; the ratio for mice that received N-hydroxy-AB as compared to those given AB was 1.7. The average numbers of tumors per liver were similar for the mice given injections of N-hydroxy-MAB, N-hydroxy-EAB, N-hydroxy-AB, and MAB. 2-Aminoazulene was inactive in this test.

**DISCUSSION**

The requirement of most chemical carcinogens for metabolism *in vivo* to proximate and ultimate carcinogenic derivatives for the induction of tumors is now generally recognized (34, 44). A proximate carcinogen is both a metabolite of a chemical carcinogen and a precursor of an ultimate reactive metabolite. In principle, proximate and ultimate chemical carcinogens should exhibit greater carcinogenic activities in a wider variety of tissues than do the parent carcinogens, since one or more

---

Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Av. wt gain at rats 2 mos.</th>
<th>No. of rats alive at 19 mos.</th>
<th>No. of rats at injection site by 10 mos.</th>
<th>No. of rats with hepatocellular carcinoma by 19 mos.</th>
<th>Other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Benzoyloxy-MAB</td>
<td>79</td>
<td>0</td>
<td>13</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>N-Hydroxy-MAB</td>
<td>97</td>
<td>18</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>MAB</td>
<td>131</td>
<td>19</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>N-Hydroxy-EAB</td>
<td>124</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>EAB</td>
<td>126</td>
<td>19</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>N-Hydroxyl-N-methyl aniline</td>
<td>122</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-Methylaniline</td>
<td>126</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>(Trioctanoin only)</td>
<td>134</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* In addition, about one-third of the rats (including those in the solvent control group) that survived to 18 to 19 months had interstitial cell tumors of the testes.

Incidence significantly greater (p < 0.001) than that for the solvent control group.

---

Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>µmol/g body wt./dose</th>
<th>No. of mice weaned</th>
<th>No. of tumor-bearing mice (%)</th>
<th>Av. no. of tumors/mouse</th>
<th>No. of mice with other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Hydroxy-MAB</td>
<td>0.4</td>
<td>48</td>
<td>35</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>MAB</td>
<td>0.4</td>
<td>55</td>
<td>55</td>
<td>89</td>
<td>9</td>
</tr>
<tr>
<td>N-Hydroxy-EAB</td>
<td>0.3</td>
<td>33</td>
<td>33</td>
<td>86</td>
<td>3</td>
</tr>
<tr>
<td>EAB</td>
<td>0.3</td>
<td>36</td>
<td>33</td>
<td>86</td>
<td>1</td>
</tr>
<tr>
<td>(Trioctanoin only)</td>
<td></td>
<td>43</td>
<td>35</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Hydroxy-MAB</td>
<td>0.3</td>
<td>40</td>
<td>35</td>
<td>86</td>
<td>5</td>
</tr>
<tr>
<td>MAB</td>
<td>0.3</td>
<td>51</td>
<td>45</td>
<td>84</td>
<td>5</td>
</tr>
<tr>
<td>N-Hydroxy-EAB</td>
<td>0.3</td>
<td>44</td>
<td>40</td>
<td>90</td>
<td>7</td>
</tr>
<tr>
<td>EAB</td>
<td>0.3</td>
<td>50</td>
<td>42</td>
<td>84</td>
<td>3</td>
</tr>
<tr>
<td>N-Hydroxy-AB</td>
<td>0.3</td>
<td>42</td>
<td>37</td>
<td>84</td>
<td>6</td>
</tr>
<tr>
<td>AB</td>
<td>0.3</td>
<td>45</td>
<td>41</td>
<td>83</td>
<td>3</td>
</tr>
<tr>
<td>2-Aminoazulene</td>
<td>0.3</td>
<td>53</td>
<td>43</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>(Trioctanoin only)</td>
<td></td>
<td>47</td>
<td>42</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Uninjected</td>
<td></td>
<td>46</td>
<td>44</td>
<td>32</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data are tabulated for mice living 9 to 11 months in Experiment 1 and 9 to 12 months in Experiment 2.

Incidence significantly greater (p < 0.05) than those for the appropriate solvent control group.

---

Downloaded from cancerres.aacrjournals.org on May 1, 2017. © 1979 American Association for Cancer Research.
metabolic activation and inactivation steps have been bypassed. However, instability, reactivity, solubility, transport, and other pharmacodynamic parameters may so limit the access of the administered proximate or ultimate carcinogen to critical cellular targets that enhanced activity is not observed. Accordingly, externally administered proximate and ultimate carcinogens may exhibit activities either above or below those of the parent precarcinogens. For instance, the proximate carcinogen N-hydroxy-2-acetylamino-9-fluorenone is a stable and relatively unreactive metabolite, and its greater carcinogenicity as compared to that of the parent amide is readily demonstrable (27, 28). However, 2-acetylamino-9-fluorenone-N-sulfate (N-sulfon-oxy-2-acetylamino-9-fluorenone), a probable major ultimate carcinogen in rat liver (9, 30, 45), is so reactive that attempts to demonstrate its carcinogenic activity on administration to the rat have been unsuccessful (2). The analogous, but less reactive, esters N-acetoxy- and N-myristoyloxy-2-acetylaminofluorenene are highly carcinogenic in this species (2, 33).

Metabolic data suggest that MAB-N-sulfate may be an ultimate carcinogenic metabolite of MAB in the rat liver. As yet, this compound has not been synthesized for direct tests, but like 2-acetamidofluorenene-N-sulfate it will probably prove to be very unstable in aqueous media. Some of the properties of N-hydroxy-MAB, especially its instability, also make assessment of its carcinogenic activity difficult. In aqueous solution, N-hydroxy-MAB is easily oxidized nonenzymatically by molecular oxygen to an unstable nitroxine, which decomposes to several products (17). By analogy to the reduction of N-hydroxy-2-acetylamino-9-fluorenone to 2-acetylamino-9-fluorenone in rats (11, 24), N-hydroxy-MAB may also be reduced to MAB in vivo. These and any other metabolic inactivation reactions which may occur in vivo would reduce the effective dose of administered N-hydroxy-MAB and could thus account for the observation that N-hydroxy-MAB was usually no more active than MAB for the induction of hepatic tumors in rats and mice (Tables 1, 2, and 4).

The development of carcinomas and multiple papillomas of the forestomach in rats intubated with N-hydroxy-MAB, but not in rats given MAB, clearly supports the role of N-hydroxy-MAB as a proximate carcinogen (Table 1). Administration to rats of N-hydroxy-2-acetylamino-9-fluorenone and of N-hydroxy-7-fluoro-2-acetylamino-9-fluorenone p.o. has similarly resulted in the development of carcinomas and multiple papillomas of the forestomach, where the parent amides were inactive (26, 28). Likewise, although the incidences were low and of marginal statistical significance, N-hydroxy-MAB yielded more sarcomas at the s.c. or i.p. injection sites of rats than did MAB (Tables 2 and 3). N-Hydroxy-MAB was much less active in the induction of sarcomas than was the synthetic ultimate carcinogen N-benzoxyloxy-MAB.

Although the induction of hepatic tumors in rats by the aminoaoo dyes has conventionally required their administration over periods of weeks to months (32), the present data indicate that the neoplastic change can be initiated rapidly by MAB. Thus, 5 of 30 partially hepatectomized rats that were given a single large dose of MAB with subsequent long-term administration of phenobarbital developed hepatocellular carcinomas (Table 2). Similar studies with diethylnitrosamine suggest that the administration of the carcinogen during the period of DNA replication facilitates the initiation of neoplastic cells and that the long-term administration of phenobarbital promotes the growth of altered hepatic cell populations (38). The promotion of the carcinogenic activities of 2- and 3'-methyl-N,N-dimethyloxy-4-aminoazobenzene for the liver of the rat by prolonged treatment with phenobarbital was shown earlier, but in the latter cases the dyes were administered over at least a 3-week period (19, 20).

The much lower carcinogenicities of EAB and N-hydroxy-EAB in the rat in these experiments, as compared to the carcinogenicities of MAB and N-hydroxy-MAB (Table 1), reflect the well-documented difference in the carcinogenicities of N- and N-ethyl derivatives of AB in this species (1, 31, 42). Similarly, the inactivity of N-hydroxy-AB in these experiments is consistent with our earlier negative data on tumor induction by N-hydroxy-AB (41) and with the observations that AB has very little carcinogenic activity in the rat (14, 32). These lower carcinogenic activities are presumably related to differences in the metabolism and reactivities of the dyes and their metabolites. Although EAB and AB are N-hydroxylated by rat liver microsomes (at rates 140 and 60%, respectively, of those of MAB), the rates of O-sulfonation of N-hydroxy-EAB and of N-hydroxy-AB by 3'-phosphoadenosine 5'-phosphosulfate-fortified liver cytosol from male rats are only 50 and 25%, respectively, of that for N-hydroxy-MAB (18). In addition, N-hydroxy-MAB readily reacts nonenzymatically, presumably through the intermediate formation of a nitroxine, with compounds that contain carbon-carbon or carbon-nitrogen double bonds (17). N-Hydroxy-EAB has much less reactivity under the same conditions.

In contrast to the observations for rat liver, AB, EAB, MAB, and their N-hydroxy derivatives all induced malignant hepatic neoplasms (types A and B) in mice treated prior to weaning (Table 4). The activities of AB, N,N-dimethyl-4-aminoazobenzene, and 2- and 3'-methyl-N,N-dimethyl-4-aminoazobenzene under similar conditions have been reported by Roe et al. (40). In our experiments, MAB was more active than either AB or EAB in the induction of hepatic tumors, but N-hydroxy-AB and N-hydroxy-EAB were each at least as active as N-hydroxy-MAB. N-Hydroxy-EAB and N-hydroxy-AB are each chemically more stable in aqueous solution than is N-hydroxy-MAB (17). The capabilities of these preweanling mice for the metabolism of the aminoaoo dyes have not been investigated.

The injection of chemicals into male mice prior to weaning appears to be a sensitive assay for the carcinogenic potential of chemicals (4, 10, 40, 43). It seems most reasonable to consider that under these conditions the chemicals act primarily as initiators. The replication of the liver during normal growth, together with possible endogenous promoters (e.g., androgens), provide the initiating stimulus. Although the occurrence of spontaneous hepatomas in these male mice may be a concern in the use of the assay, this susceptibility may also be a factor in its sensitivity. Thus, the development of hepatomas in mice could be analogous to the model developed by Knudson et al. (21) for human retinoblastoma, i.e., that the induction requires 2 mutations and that highly susceptible individuals carry one mutation in the germ line. However, it is evident that an increase in the probability of the occurrence of any step in a multistep model for the induction of cancer would increase the ultimate tumor incidence.

ACKNOWLEDGMENTS

The authors are indebted to Lona Barsness, Mary Kolstad, and Nancy Korda for their assistance with these experiments.
REFERENCES


The N-Hydroxy Metabolites of N-Methyl-4-aminoazobenzene and Related Dyes as Proximate Carcinogens in the Rat and Mouse

Elizabeth C. Miller, Fred F. Kadirabar, James A. Miller, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/39/9/3411

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.