On the Protective Action of Certain Polycyclic Aromatic Hydrocarbons against Carcinogenesis by Aminoazo Dyes and 2-Acetylaminofluorene*

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Attempts to induce liver tumors with the carcinogenic polycyclic aromatic hydrocarbons have generally failed (9). However, certain of these hydrocarbons have a marked effect on hepatic carcinogenesis induced by other agents. Thus, Richardson et al. (32) observed that 3-methylcholanthrene1 (MC), administered at a low level to rats also receiving the strong hepatocarcinogen 3'-methyl-4-dimethylaminoazobenzene (3'-methyl-DAB), strongly retarded the induction of liver tumors. This finding has been confirmed and extended by Meechan et al. (12) and by the experiments reported in this paper. The carcinogenicity of 2-acetylaminofluorene (AAF) in the liver and at other sites is similarly inhibited by the simultaneous administration of low levels of certain hydrocarbons (10, 27, and this paper).

The purpose of this report is to compare the effects of various hydrocarbons on tumor induction by 3'-methyl-DAB and to show that the carcinogenic activities of certain other aminoazo dyes and of AAF and its 7-fluoro derivative are also strongly inhibited by MC. Data are presented on the levels of free and protein-bound aminoazo dyes and on the azo dye N-demethylase and reductase activities of the livers of rats fed 3'-methyl-DAB alone or with various hydrocarbons. These data suggest that the hydrocarbons inhibit azo dye carcinogenesis through the maintenance of high levels of certain enzyme systems which metabolize the dye to noncarcinogenic derivatives so that less dye is available for metabolism along routes leading to carcinogenesis. Presumably, a similar mechanism is involved in the inhibition of tumor induction by AAF.

MATERIALS AND METHODS

Care of the animals.—Young adult albino rats,² with initial weights of 180–230 gm., were used throughout. The animals were housed in screen-bottomed cages in groups of four. In the experiment with AAF, both sexes were used and caged separately; in all other experiments only male rats were studied. The diets and water were available ad libitum, and the animals were weighed monthly.

In the experiments on tumor induction by the aminoazo carcinogens the dyes, with or without one of the polycyclic hydrocarbons, were routinely fed for 13 weeks; at this time the rats were laparotomized, and the livers were examined for tumors. The rats were then fed the same diet without dye or hydrocarbon for an additional 2 months to allow any small tumors to grow to a recognizable size before the rats were killed for a final tumor incidence. As indicated below, in some experiments additional animals were fed each of the various diets, and groups of three rats were taken at intervals for analytical studies.

3'-Methyl-DAB (8) was incorporated in a diet composed of crude casein, 180 gm.; Cerelose₃ (glucose monohydrate), 710 gm.; corn oil, 50 gm.; Vitab⁴ (rice bran extract), 20 gm.; salt mixture (13), 40 gm.; Mead's percomorphum oil, 300 mg.; and riboflavin, 2 mg. (including that in the crude casein and Vitab). 3'-Methyl-DAB was generally fed as 0.054 per cent of the diet, but specified groups were fed 0.027 or 0.108 per cent of this dye. 4'-Fluoro-DAB (20) and 2',4'-difluoro-DAB (28) were fed in a diet containing crude casein, 120 gm.; Cerelose, 770 gm.; and riboflavin, 2 mg. (including that in the crude casein and Vitab).

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¹ This compound has also been designated 20-methylcholanthrene.

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corn oil, 50 gm.; Vitab, 20 gm.; salt mixture, 40 gm.; p-toluidine, 300 mg.; and riboflavin, 1 mg. (including that in the casein and Vitab). The 8'-fluoro-DAB and 8',4'-difluoro-DAB were fed at levels of 0.059 and 0.068 per cent, respectively, for the first 3 weeks. At this time the rats not receiving MC were in poor condition, they were transferred to the basal diet for 4 days and then given 0.059 and 0.059 per cent, respectively, of these dyes for the remaining 16 weeks. At the end of the experiment the dyes were dissolved in the corn oil of the diet with mild heat.

AAF (25), with or without MC, was fed at 0.08 per cent of a grain diet (25) for 16 weeks. 7-Fluoro-AAP (25), alone or with MC, was incorporated in the same diet at a level of 0.02 per cent; this diet was fed for 144 weeks. At these times the rats were transferred to the same diet without either of the fluorine derivatives or MC. At 28 weeks the livers of the rats were examined for tumors by laparotomy, and the rats were killed for a final tumor count at 32 weeks. Both AAF and 7-fluoro-AAP were added to the diets as 10 gm. of a glucose triturate/kg.

The polyacrylamide hydrocarbons and the 9,10-dimethyl-1,2-benzanthracene photooxide (8) were added to the diets as solutions in either acetone or ethyl ether, and the solvents were allowed to evaporate at room temperature. The diets were then thoroughly mixed. The final concentration of the hydrocarbons was 0.123 millimoles/kg (0.0088 per cent MC) in the experiments with 3'-methyl-DAB and 0.184 millimoles/kg (0.0086 per cent) for all other experiments. Analytical procedures.—In general, three representative rats were selected from each group on the basis of body weight. In Series II, in which the level of 3'-methyl-4-aminoazobenzene (3'-methyl-AB) in the blood was determined, 0.75–1.5 ml. was withdrawn from the heart into a heparinized 2-ml. syringe while the rats was under ether anesthesia. The volume was recorded, and the blood was expelled into the assay tube containing the alkaline required for digestion (10). All the rats of Series II were killed by decapitation, the livers were exposed, and the median lobes were rapidly ligated and removed. These samples for the N-demethylase and reducative assays were immediately chilled in beakers of ice-cold 1.15 per cent KCl. The remaining liver in each case was perfused with ice-cold 0.85 per cent sodium chloride. Because of technical limitations the livers of only two of the three rats killed at each time were assayed for the two enzyme systems, but all three livers were assayed for bound dye and riboflavin. The animals of the other series were killed with ethyl ether, and the entire liver was perfused with ice-cold sodium chloride solution. In all cases the perfused livers were immediately transferred to ice-cold beakers, weighed, and aliquots taken for the various analyses.

For the determination of protein-bound dye a 15 per cent suspension of each liver was prepared in a pestle-type homogenizer, and the protein was precipitated with trichloroacetic acid at a final concentration of 9 per cent. The protein was then extracted, dried, and analyzed for total protein-bound dye (14, 18). The dye solutions were read in a Lumetron colorimeter equipped with a 515-mµ filter, and the values are expressed as the absorbance per 100 mg. of dried protein after correction for the average absorbance (0.04/100 mg.) of extracts of normal rat liver protein prepared and analyzed in the same manner.

The amount of 3'-methyl-AB in the blood was determined as described previously for 4-aminoazobenzene (19), except that the readings were made with a Lumetron colorimeter equipped with a 515-mµ filter. The free dyes in the liver were determined on pooled samples composed of 0.67 gm. from each of three livers by the method previously described for the determination of 4-aminoazobenzene and its N-methyl derivatives (19). The readings were made in a Cenco-Sheard colorimeter at 508, 514, and 560 mµ for 3'-methyl-AB, 3'-methyl-4-monomethylazobenzene (3'-methyl-MAB), and 3'-methyl-DAB, respectively. Under the conditions of these analyses 69, 84, and 90 per cent, respectively, of 3'-methyl-AB, 3'-methyl-MAB, and 3'-methyl-DAB which were added to normal liver could be recovered; in each case the values reported have been corrected by these amounts.

Riboflavin was determined by the method of Conner and Straub (4) after neutralization of the acid hydrolysates with sodium acetate and incubation with papain for 2 hr. at 37° C.

The demethylase assay was carried out as described by Mueller and Miller (30), except that the 3-methyl-MAB (150 µg/flask) was added in 0.1 ml. of methanol rather than ethanol. Fifty mg. of liver was added per flask, and the incubation time was 30 minutes. The reductase assay of Mueller and Miller (29) was used with 150 µg. of DAB and 30 mg. of liver per flask. Hexose diphosphate, rather than glucose-6-phosphate, was included in the reaction medium, and the incubation time was 30 min.

RESULTS

The effects of various hydrocarbons on tumor induction by aminoazo dyes.—As reported by Richardson et al. (32), the addition of 0.0038 per cent of MC to a diet containing 0.054 per cent of 3'-methyl-DAB strongly inhibited the induction of liver tumors in rats. In our experiments (Table 1), 48–75 per cent of the rats fed 0.054 per cent of 3'-methyl-DAB without any hydrocarbon had tumors at 3 months; the tumor incidence was 88–100 per cent after an additional 2 months on the basal diet (Groups 1, 7, 12, 16, 19). In contrast, none of the rats fed MC with this level of dye had tumors at 3 months, and after 2 months on the basal diet tumors were found in only two of the 62 rats (Groups 2, 8, 13, 20). The inhibitory effect of MC was not altered by the addition of 0.5 per cent of L-cystine to the diet (Groups 5 and 6). When incorporated in the diet at the same molar level, 3,4-benzpyrene and 1,2,5,6-dibenzanthracene (Groups 3 and 4) were as effective as MC in protecting the rats against the induction of liver tumors. The incidence of tumors when any of these three hydrocarbons was fed with 0.054 per cent of dye was similar to that obtained on feeding 0.027 per cent of 3'-methyl-DAB in the absence of any hydrocarbon (Group 11). 1,2-Benzanthracene (Group 9) gave moderately good protection; there were no tumors at 3 months and only a 40 per cent incidence at 5 months. 9,10-Dimethyl-1,2-benzanthracene and its photooxide (Groups 14, 15, 17, and 18), although much less effective, also re-
tarded the development of tumors. Pyrene (Group 10) was without effect on tumor induction.

In one experiment (Series V), MC was fed in a diet containing twice the usual level of 3'-methyl-DAB. Both in terms of toxicity (weight gain) and of carcinogenicity, 0.108 per cent of 3'-methyl-DAB administered simultaneously with 0.0033 per cent of MC was approximately equivalent to 0.054 per cent of the dye when it was fed alone (Groups 19 and 20). The excellent survival of the rats fed the high level of dye with MC was particularly striking, since rats fed this level of dye without any hydrocarbon died within 3–4 weeks.

MC exerted a protective effect only when given simultaneously with 3'-methyl-DAB. No inhibition was obtained when the diet containing 0.0033 per cent of MC was fed for 4 weeks prior to or for 12 weeks following the administration of 0.054 percent of 3'-methyl-DAB in the diet for 8 or 10 weeks. Thus, the final tumor incidence for the control rats fed the dye for 10 weeks was 83 per cent, as compared with incidences of 78 per cent for those prefed the MC-containing diet for 4 weeks or 94 per cent for those subsequently fed the diet containing MC for 12 weeks. When the dye was fed for only 8 weeks, the tumor incidences were 53 and 50 per cent, respectively, for those subsequently fed either the basal diet alone or the basal diet plus MC.

The induction of liver tumors by 4'-fluoro-DAB and 2',4'-difluoro-DAB was likewise retarded by the simultaneous administration of MC, although the inhibition was not as great as in the experiments with 3'-methyl-DAB (Table 2).

Regardless of the dyes or hydrocarbons employed, there was a close parallelism between the inhibition of tumor development, retardation of the development of other gross liver damage (cirrhosis), and increased weight gains. In all the groups which were completely protected from tumor development, liver damage was minimal. Approximately 90 per cent of the rats which did not develop gross tumors survived until the experiment was terminated at 5 months.

The inhibition by MC of tumor induction by 2-acetylaminofluorene and its 7-fluoro derivative.—A similar strong inhibition of tumor development was observed when MC was fed with 2-acetyl-

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**Table 1**

<table>
<thead>
<tr>
<th>Series</th>
<th>Group</th>
<th>3'-Me-DAB (per cent)</th>
<th>Compounds added to diet*</th>
<th>Wt. increment at 1 mo. (gm.)</th>
<th>No. Rats at start</th>
<th>No. of Rats with liver tumors 3 mo.</th>
<th>Of No. w/ Gross cirrhosis</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>0.054</td>
<td>None</td>
<td>9</td>
<td>8, 16</td>
<td>15</td>
<td>Moderate-severe</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>3-Methylcholanthrene</td>
<td>24</td>
<td>16</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>3,4-Benzo-pyrene</td>
<td>27</td>
<td>16</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>1,2,5,6-Dibenzanthracene</td>
<td>35</td>
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<td>0</td>
<td>None</td>
</tr>
<tr>
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<td>5</td>
<td></td>
<td>0.5% Cystine</td>
<td>7</td>
<td>11</td>
<td>5</td>
<td>Moderate-severe</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td>0.3% Cystine, 3-methylcholanthrene</td>
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<td>12</td>
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<td>None</td>
</tr>
<tr>
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<td>7</td>
<td>0.064</td>
<td>None</td>
<td>1</td>
<td>16</td>
<td>8</td>
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<tr>
<td></td>
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<td></td>
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<td>None</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td></td>
<td>1,2-Benzanthracene</td>
<td>30</td>
<td>18</td>
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</tr>
<tr>
<td></td>
<td>10</td>
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<td>Pyrene</td>
<td>-16</td>
<td>16</td>
<td>6</td>
<td>Moderate-severe</td>
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<td></td>
<td>11</td>
<td>0.087</td>
<td>None</td>
<td>17</td>
<td>16</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
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<td>12</td>
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<td>-10</td>
<td>14</td>
<td>9</td>
<td>Severe</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td></td>
<td>3-Methylcholanthrene</td>
<td>30</td>
<td>17</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td></td>
<td>9, 10-Dimethyl-1,2-benzanthracene</td>
<td>20</td>
<td>17</td>
<td>5</td>
<td>Mild-moderate</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td></td>
<td>9,10-Dimethyl-1,2-benzanthracene photo-oxide</td>
<td>25</td>
<td>17</td>
<td>3</td>
<td>Mild-moderate</td>
</tr>
<tr>
<td>IV</td>
<td>16</td>
<td>0.054</td>
<td>None</td>
<td>-11</td>
<td>14</td>
<td>8</td>
<td>Severe</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td></td>
<td>9, 10-Dimethyl-1,2-benzanthracene</td>
<td>38</td>
<td>17</td>
<td>3</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td></td>
<td>9,10-Dimethyl-1,2-benzanthracene photo-oxide</td>
<td>17</td>
<td>17</td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td>V</td>
<td>19</td>
<td>0.054</td>
<td>None</td>
<td>0</td>
<td>16</td>
<td>7</td>
<td>Moderate-severe</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
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<td>52</td>
<td>16</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0.108</td>
<td>None</td>
<td>3</td>
<td>16</td>
<td>5</td>
<td>Moderate-severe</td>
</tr>
</tbody>
</table>

* Each of the hydrocarbons was added to the diet at a level of 0.123 millimoles/kg.
aminofluorene; marked protection was obtained at each of the four major sites of tumor induction in these rats (Table 3, Groups 1 and 2). Of the male rats fed AAF alone, 73 per cent developed liver tumors, 37 per cent carcinomas of the ear ducts, and 10 per cent adenocarcinomas of the small intestine. By contrast, the incidences of tumors at each of these sites were 0, 3, and 0 per cent among male rats fed AAF and MC simultaneously. Of the female rats fed AAF alone, 47 per cent developed carcinomas of the ear duct, 73 per cent carcinomas of the mammary gland, and 7 per cent adenocarcinomas of the small intestine. Only 14 per cent of the female rats fed AAF and MC together had adenocarcinomas of the mammary gland, and no other tumors were noted.

MC likewise markedly protected rats fed 7-fluoro-AAF against its toxic and carcinogenic actions (Table 3, Groups 3 and 4). When 0.02 per cent of 7-fluoro-AAF was fed to male rats, only seven of the eighteen survived until 23 weeks; all the survivors had liver tumors and severe liver damage at this time. Two of these seven rats also had carcinomas of the ear duct and one an adenocarcinoma of the small intestine. Inclusion of 0.0036 per cent of MC in the diet containing 7-fluoro-AAF permitted the survival of seventeen of the eighteen male rats until 23 weeks, with an average weight gain of 136 gm. None of these rats had tumors at 23 weeks, and only 24 per cent had developed liver tumors by 32 weeks when the experiment was terminated. Only mild liver damage was observed in this group.

Rats fed 0.0036 per cent of MC alone gained considerable weight, survived well, and developed no gross neoplasms.

The effects of various hydrocarbons on the levels of free and bound dyes in rats fed 3'-methyl-DAB.—The protection afforded by certain hydrocarbons against the induction of liver tumors by 3'-methyl-DAB was paralleled by decreased levels of free and protein-bound dye in the liver and of free dye in the blood of rats fed these hydrocarbons. Conversely, those hydrocarbons which were less

### TABLE 2

THE INHIBITION BY S-METHYLCHOLANTHRENE OF HEPATIC TUMOR INDUCTION WITH 4'-FLUORO- AND 2',4'-DIFLUORO-4-DIMETHYLAMINOAZOBENZENE

<table>
<thead>
<tr>
<th>GROUP</th>
<th>COMPOUNDS ADDED TO DIET*</th>
<th>NO. RATS WITH LIVER TUMORS</th>
<th>NO. RATS WITH GROSS CIRRHOSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4'-Fluoro-DAB</td>
<td>55</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>4'-Fluoro-DAB+MC</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>2',4'-Difluoro-DAB</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>2',4'-Difluoro-DAB+MC</td>
<td>60</td>
<td>10</td>
</tr>
</tbody>
</table>

* 4'-Fluoro-DAB and 2',4'-difluoro-DAB were fed as 0.059 and 0.059 per cent, respectively, of the diet for the first 5 weeks. The rats were then fed the basal diet for 4 days, after which the dyes were included in the diets for 10 weeks at levels of 0.053 and 0.059 per cent, respectively. MC was added as 0.0036 per cent of the diet.

### TABLE 3

THE INHIBITION BY S-METHYLCHOLANTHRENE OF TUMOR INDUCTION BY 2-ACETYLAMINOFLUORENE AND 7-FLUORO-2-ACETYLAMINOFLUORENE

<table>
<thead>
<tr>
<th>GROUP</th>
<th>COMPOUNDS ADDED TO DIET*</th>
<th>NO. OF RATS WITH TUMORS IN:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAF</td>
<td>No. of rats fed to diet 16</td>
</tr>
<tr>
<td>2</td>
<td>AAF+MC</td>
<td>No. of rats fed to diet 16</td>
</tr>
<tr>
<td>3</td>
<td>7-Fluoro-AAF</td>
<td>No. of rats fed to diet 16</td>
</tr>
<tr>
<td>4</td>
<td>7-Fluoro-AAF+MC</td>
<td>No. of rats fed to diet 16</td>
</tr>
<tr>
<td>5</td>
<td>MC</td>
<td>No. of rats fed to diet 16</td>
</tr>
</tbody>
</table>

* AAF and 7-fluoro-AAF refer to 2-acetylaminofluorene and its 7-fluoro derivative; these were fed as 0.08 and 0.02 per cent of the grain diet, respectively. MC was added as 0.0036 per cent of the diet.
† Alive at 32 weeks plus those dead with a tumor prior to 23 weeks.
‡ The data for Groups 1 and 2 are the combined results of two experiments.
inhibitory to carcinogenesis by 3'-methyl-DAB had smaller effects on the levels of dye in the liver and blood. Thus, as shown in Chart 1, at 18 days the livers of rats fed 0.054 per cent of dye with MC contained less than one-half as much 3'-methyl-DAB, 3'-methyl-MAB, and 3'-methyl-AB as those fed the same level of dye alone and about the same amount as those fed only one-half as much 3'-methyl-DAB. Pyrene caused no reduction in the level of free dye in the liver, and 1,2-benzanthracene produced an intermediate effect. Similar differences between these groups were seen at 33 days. Likewise, from the 5th to the 33d day the level of 3'-methyl-AB in the blood of rats fed 0.054 per cent of 3'-methyl-DAB with MC was 60 per cent or less of that found in rats fed this level of dye alone and about the same as that of rats fed 0.027 per cent of dye. Pyrene had no effect on the level of 3'-methyl-AB in the blood, while 1,2-benzanthracene caused a marked reduction by 33 days, but not at earlier times.

![Chart 1](attachment:chart1.png)

**Chart 1.**—The levels of 3'-methyl-DAB, 3'-methyl-MAB, and 3'-methyl-AB in the livers and of 3'-methyl-AB in the blood of rats fed 3'-methyl-DAB alone or with certain hydrocarbons (Series II). Each value for liver was obtained from a single analysis of a composite sample from three livers. For the data on blood each bar represents the average of individual analyses on the blood from three rats; the ranges are indicated by the narrow vertical lines. PY = pyrene; BA = 1,2-benzanthracene; and MC = 3-methylcholanthrene.

Administration of MC, 3,4-benzpyrene, 1,2,5,6-dibenzanthracene, or 1,2-benzanthracene with 0.054 per cent of 3'-methyl-DAB caused a marked alteration in the pattern of the protein-bound dye levels when they were plotted as a function of the time of dye-feeding (Chart 2). When these hydrocarbons were fed, there was a less rapid rise in the level of protein-bound dye during the first 2 weeks than that observed in control rats fed the same level of dye without any hydrocarbon. The maximum level of bound dye was reached in all of the groups at 2–3 weeks. Thereafter, the level of protein-bound dye in the livers of the control rats fed 0.054 per cent of 3'-methyl-DAB fell gradually to 50–60 per cent of the maximum value, while the level in the livers of the rats fed the protective hydrocarbons remained essentially constant. The bound dye pattern for the livers of rats fed 0.027 per cent of 3'-methyl-DAB was similar to that of rats fed 0.054 per cent of dye with a hydrocarbon protecting against tumor development, while the pattern for rats fed 0.054 per cent of dye with pyrene, which did not inhibit carcinogenesis, was similar to that of rats fed this level of dye alone. Similarly, in another experiment the bound dye levels at 1, 2, and 5 weeks for the livers of rats fed 0.108 per cent of 3'-methyl-DAB with 0.0033 per cent of MC were nearly identical to those for rats fed 0.054 per cent of the dye without the hydrocarbon. These results are in line with the high incidence of liver tumors which was obtained when 0.108 per cent of this dye was fed with MC (Table 1).

The observed bound dye levels represent a balance between the formation and degradation of this metabolite (15). To determine on which of these processes the hydrocarbons were acting, rats were fed 0.054 per cent of 3'-methyl-DAB with or
without 0.0033 per cent of MC for 18 days, starved for 12 hours, and then transferred to the corresponding basal diets. The short period of starvation was interposed to allow for the absorption of most of the dye from the gastrointestinal tract. The rats on each basal diet (with or without MC) were restricted in their food consumption to a level (about 85 per cent of ad libitum) which permitted maintenance of body and liver weights at approximately the same levels as at the end of the dye-feeding period. Groups of three rats were killed at the end of the dye feeding period and after 2, 4, and 7 days on the basal diets for the determination of protein-bound dye in the liver. In this experiment the half-life of the bound dye, whether or not MC was fed, was about 3 days. It therefore appears that the administration of MC diminishes the formation of the bound derivative of 3'-methyl-DAB rather than hastening its degradation.

Effect of 3'-methyl-DAB alone or with various hydrocarbons on hepatic N-demethylase and reductase activities.—When rats previously fed the stock laboratory diet were transferred to the semipurified diet, there was a general decrease in the hepatic levels of the reductase and N-demethylase systems (Chart 3). However, the extent of the decreases was dependent on the diet fed and paralleled the tumor incidences in comparable rats maintained on these diets for longer periods (Table 1, Series II). Thus, the livers of rats fed 0.054 per cent of 3'-methyl-DAB alone or with pyrene showed the greatest decreases in reductase and demethylase activities; all the animals fed these diets for 3 months developed tumors by 5 months. The livers of the animals fed the basal diet with or without MC generally had the highest enzyme activities at all times of assay, while the livers of the rats fed diets which greatly retarded tumor formation had demethylase and reductase levels approaching those of the rats on the basal diets. This group included the rats fed 0.027 per cent of 3'-methyl-DAB and those fed 0.054 per cent of 3'-methyl-DAB with either 1,2-benzanthracene or MC.

The demethylase system was more labile to the feeding of 3'-methyl-DAB than the reductase system. Thus, after 33 days the livers of the rats fed 0.054 of 3'-methyl-DAB had only 25 per cent as much demethylase activity as the rats fed the basal diets, while their reductase activities were 50 per cent of those of the rats on the basal diets. The livers of the rats fed 0.054 per cent of 3'-methyl-DAB with MC had approximately 75 per cent as much of each enzyme activity as the animals on the basal diets.

Effect of 3'-methyl-DAB alone or with various hydrocarbons on hepatic riboflavin.—As in our previous studies (17), administration of 3'-methyl-DAB caused a loss of hepatic riboflavin, although the extent of the decrease was less with these rats fed an 18 per cent casein diet than in earlier work with a 12 per cent casein diet. Thus, from an initial level of 19 μg/gm of liver, the hepatic riboflavin dropped by the 18th day to 16 μg/gm in the basal-fed rats, whether or not MC was included in the diet, and to 18, 15, 14, and 16 μg/gm for the rats fed 0.054 per cent of 3'-methyl-DAB alone or with MC, pyrene, or 1,2-benzanthracene, respectively. The level in the livers of rats fed 0.027 per cent of 3'-methyl-DAB was 17 μg/gm. At 39 days the corresponding figures were 18 μg/gm for the rats on the basal and basal plus MC diets and 10, 15, 14, 16, and 17 μg/gm for the dye-fed rats listed in the same order as above. Thus, while the rats fed 0.054 per cent of 3'-methyl-DAB alone had lower levels of hepatic riboflavin than the rats fed this level of dye with MC, the hepatic riboflavin was comparable in rats fed the dye with any of the three hydrocarbons studied, even though they differed markedly in their abilities to protect against liver tumor induction by 3'-methyl-DAB.
DISCUSSION

The studies of Miyaji et al. (27) and those described in this paper have demonstrated that the inhibition of carcinogenesis by MC and related polycyclic aromatic hydrocarbons is not specific for the hepatocarcinogen 3'-methyl-DAB, but that inhibition also occurs when these hydrocarbons are fed with other aminoazo dyes or with AAF and its 7-fluoro derivative. An important problem was to determine the mechanism by which these hydrocarbons conferred protection. The lower levels of free and bound dyes in the liver and of 3'-methyl-AB in the blood of rats fed 3'-methyl-DAB with a protective hydrocarbon suggested that the metabolism of the dye was more rapid in the hydrocarbon-treated rats and that less dye was available for the reactions leading to carcinogenesis. Assays for the reductase and demethylase activities of the livers from rats fed dye alone or with a hydrocarbon demonstrated that this was the case. The reductase system reduces 3'-methyl-DAB and its N-demethylated derivatives to m-toluidine and p-phenylene diamine or one of its N-methyl derivatives, none of which has proved carcinogenic to rats (21, 24). Similarly, the ultimate product of the demethylase reaction, 3'-methyl-AB, is much less active in inducing tumors than its N-methyl derivatives (22). After 1 month, liver homogenates from rats fed 3'-methyl-DAB with a protective hydrocarbon had almost as much of each of these systems as liver homogenates from rats on the basal diet; however, the livers from rats fed the same level of dye without hydrocarbon had but one-fourth as much demethylase activity and one-half as much reductase activity. Both in terms of tumor incidence and metabolic data, the feeding of 0.054 per cent 3'-methyl-DAB with 0.0033 per cent MC was equivalent to the feeding of one-half as much dye in the absence of hydrocarbon. Comparable metabolic studies were not made on the livers of rats fed the fluorinated aminoazo dyes, but the situation would be expected to be similar.

The reductase and demethylase systems presumably do not function in the metabolism of AAF; however, as the Weisburgers (34) have shown, this compound is subject to hydroxylation in vivo at several sites on the rings. Peters and Gutmann (31) and Booth and Boyland (2) have shown that metabolism of AAF to its 7-hydroxy derivative, which has little or no carcinogenic activity (1, 11), occurs in fortified rat liver homogenates and microsomes. The in vitro systems used by these authors are similar to those used in this laboratory in studies on the hydroxylation of DAB (23) and 3,4-benzpyrene (6). The activity of benzpyrene hydroxylase in liver microsomes is increased up to tenfold by the prior administration of small amounts of benzpyrene or related hydrocarbons to weanling rats (6). Recent work along the same lines in this laboratory has shown that AAF is hydroxylated at positions 1, 3, 5, and 7 by fortified rat liver homogenates and microsomes and that hydroxylation at each of these positions is greatly stimulated by MC pretreatment (7). It is possible, therefore, that increased hydroxylation to less active derivatives is the mechanism by which MC protects against the carcinogenic action of AAF. The toxicity and carcinogenicity of 7-fluoro-AAF are also markedly reduced by the administration of MC. This compound presumably is also subject to hydroxylation at the 1, 3, and 5 positions. Accelerated hydroxylation may also play a role in the protective effect of MC on the aminoazo dyes, since none of the monohydroxy derivatives of DAB has induced tumors when fed to rats (21, 24, 26, 33).

The inhibition of the loss of demethylase and reductase activities by the feeding of various hydrocarbons with 3'-methyl-DAB cannot be interpreted solely as a result of the protection of the liver from damage by the dye and a consequent retention of normal enzyme levels. Thus, in the absence of dye these hydrocarbons can cause marked increases in the activities of these liver enzyme systems. For instance, the injection of as little as 0.1 mg. of MC intraperitoneally into a weanling rat causes nearly a threefold increase in the demethylase activity 24 hours later, while the administration of 1 mg. results in a doubling of reductase activity at 24 hours and a greater than threefold increase at 72 hours (5). The properties of these systems indicated that the hydrocarbons induced the synthesis of these enzymes.

Although the most active hydrocarbon inhibitors of hepatocarcinogenesis by 3'-methyl-DAB are also very potent carcinogens when applied to the skin or subcutaneous tissue (9), the correlation between the activities of the hydrocarbons tested as carcinogens and as inhibitors of hepatocarcinogenesis was poor. Thus, the very active carcinogen 9,10-dimethyl-1,2-benzanthracene had a rather limited ability, and the very weak local carcinogen 1,2-benzanthracene had a moderately good ability to inhibit the action of 3'-methyl-DAB. There was, however, a good correlation between the ability of these hydrocarbons to cause the retention of the demethylase and reductase activities in the livers of dye-fed rats and...
to induce synthesis of the demethylase system in weaning rats (5).

In these studies a high incidence of tumors was associated with a rapid rise in the level of protein-bound dye in the liver during the first 10-15 days of dye feeding. The rise was followed during the succeeding 3 weeks by a gradual decline in the bound dye levels to 50-60 per cent of the maximum value, and this latter level was then maintained for the remainder of the dye feeding period. Under conditions which yielded either no tumors or a considerably retarded rate of induction, there was a more moderate rate of increase in the level of protein-bound dye in the early period, and at 10-15 days a plateau was reached at a level about 50-60 per cent of the maximum value observed for the high tumor groups. Thus, the initial striking difference in bound dye levels between the groups destined to develop high or low tumor incidences was obliterated after the 3d-4th week of dye feeding. A somewhat similar pattern was observed earlier in correlating the level of protein-bound dye after various times of feeding of several derivatives of DAB (18, 26). Thus, when the more carcinogenic dyes (DAB, 3'-methyl-DAB, and 4'-fluoro-DAB) were fed, the level of bound dye rose rapidly to a maximum and then declined to a lower value which was maintained to the end of the experiment. When the dyes with lesser carcinogenic activities were fed, the bound dye levels either rose rapidly to high levels which were sustained for several months (2-methyl-DAB and 3-methyl-MAB) or rose slowly to a plateau level at a later time (2' and 4'-methyl-DAB). A high incidence of tumors thus appears to be associated with a rapid rise in the level of bound dye during the first 2-4 weeks, followed by a gradual decline to a lower level. The significance of this pattern needs further study.

SUMMARY

1. The strong inhibition in the rat of hepatic carcinogenesis due to the feeding of 0.054 per cent of 3'-methyl-4-dimethylaminoazobenzene (3'-methyl-DAB) by the administration of 0.0033 per cent of 3-methylcholanthrene (MC) has been confirmed. At equimolar levels 3,4-benzpyrene and 1,2,5,6-dibenzanthracene also inhibited hepatic carcinogenesis strongly, 1,2-benzanthracene was a moderately strong inhibitor, 9,10-dimethyl-1,2 benzanthracene and its photoxide were weak inhibitors, and pyrene was without effect. One-half the level of 3'-methyl-DAB (0.027 per cent) induced few tumors in this assay in which the compounds were fed for only 3 months. The inhibitory effect of MC was overcome by increasing the level of 3'-methyl-DAB to 0.108 per cent. Administration of MC with 4'-fluoro-DAB or 2',4'-difluoro-DAB also inhibited their hepatocarcinogenic actions.

2. Administration of MC with 2-acetylaminofluorene or 7-fluoro-2-acetylaminofluorene strongly inhibited the induction in rats of tumors at the four sites (liver, mammary gland, ear duct, and small intestine) normally affected by these compounds under our conditions.

3. Metabolic studies on rats fed 3'-methyl-DAB alone or with one of the hydrocarbons indicated that the hydrocarbons protected against the induction of tumors by causing the liver to maintain high levels of certain enzyme systems which metabolize the dye to less active or inactive derivatives. Thus, administration of 0.054 per cent of 3'-methyl-DAB resulted in a marked loss of the ability of liver homogenates to N-demethylate and to reduce the azo linkage of aminoazo dyes. A similar loss of activity occurred when pyrene was fed with the dye, but not when a hydrocarbon protecting against carcinogenesis was administered. When one of the protective hydrocarbons was fed with 0.054 per cent of 3'-methyl-DAB, the levels of free aminoazo dye in the liver and blood and of protein-bound dye in the liver were similar to those found in rats fed only one-half as much dye. The levels of free and bound dye were altered to a lesser extent by the feeding of 1,2-benzanthracene and were not affected by feeding pyrene with the dye.

4. The hepatic riboflavin levels of rats fed 3'-methyl-DAB alone or with the various hydrocarbons could not be correlated with the incidences of tumors obtained under these conditions.

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