The Carcinogenicity of 3-Methoxy-4-aminoazobenzene and Its N-Methyl Derivatives for Extrahepatic Tissues of the Rat*

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SUMMARY

In contrast to all previous results with many derivatives of 4-dimethylaminoazobenzene, the ingestion by the rat of 3-methoxy-4-aminoazobenzene or its N-mono- or N,N-dimethyl derivatives was found to induce high incidences of squamous-cell carcinomas of the ear duct. 3-Methoxy-4-aminoazobenzene also induced low incidences of adenocarcinomas in the small intestine and mammary gland. In addition, the 3-methoxy dyes induced a few solitary tumors of the skin. However, these dyes exhibited very little carcinogenic activity toward the liver. No tumors were noted in rats fed 2-methoxy-4-aminoazobenzene or its N-monomethyl or N,N-dimethyl derivatives, 2-hydroxy-4-monomethylaminoazobenzene, or 2-hydroxy-4-dimethylaminoazobenzene. Syntheses are described for five new dyes: 2-methoxy-4-aminoazobenzene; 2-hydroxy-, 2-methoxy-, and 3-methoxy-4-monomethylaminoazobenzene; and 2-methoxy-4-dimethylaminoazobenzene.

The o-hydroxylation hypothesis of carcinogenesis by aromatic amines proposed by Clayson (2) and the induction of bladder tumors by the o-hydroxy metabolites of 2-naphthylamine and 4-aminobiphenyl following implantation in the mouse urinary bladder (1, 3) made it desirable to test the carcinogenicity of various hydroxyl derivatives of the hepatocarcinogenic aminoazo dyes in the rat. When none of the five possible ring monohydroxy derivatives of 4-dimethylaminoazobenzene (DAB) were found to exhibit carcinogenic activity (10, 13, 14, 19), efforts were made in this laboratory to prepare the 2- and 3-hydroxy derivatives of 4-monomethylaminoazobenzene (MAB) and of 4-aminoazobenzene (AB) in quantities adequate for test. Unfortunately, except for 2-hydroxy-MAB, facile syntheses of these dyes were not found. However, as a result of these efforts, sufficient amounts of the corresponding methoxy derivatives of DAB, MAB, and AB became available. The carcinogenicities of these compounds were determined after 1-methoxy-2-naphthylamine (3) and 3-methoxy-4-aminobiphenyl (21) were found to induce urinary bladder tumors in the mouse and rat, respectively; in the former case greater activity was observed than was found for the corresponding o-hydroxy metabolite. The unusual results of the carcinogenicity tests on the 3-methoxy dyes form the basis of this report.

MATERIALS AND METHODS

Preparation of dyes.1—The 4-aminoazobenzene (AB) was obtained from the Eastman Kodak Co. and recrystallized from a mixture of hexane and benzene. The other dyes were prepared in these laboratories. 4-Dimethylaminoazobenzene (DAB) (10), 3-methoxy-4-aminoazobenzene (3-methoxy-AB) (18), 3-methoxy-4-dimethylaminoazobenzene (3-methoxy-DAB) (13), and 2-hydroxy-4-dimethylaminoazobenzene (2-hydroxy-DAB) (14) were

1 Elementary analyses by the Huffman Microanalytical Laboratories, Wheatridge, Colorado.

Received for publication May 11, 1961.
synthesized by procedures described previously. The five dyes described below are new compounds. The melting points are uncorrected.

3-Methoxy-4-monomethylaminoazobenzene (3-methoxy-MAB).—This compound was prepared from 3-methoxy-AB. The latter dye was N-formylated as described before for MAB (14). A 70 per cent yield of dry crude N-formyl-3-methoxy-AB was obtained. Nineteen gm. (0.5 mole) of lithium aluminum hydride was suspended in 400 ml. of dry ethyl ether in a three-neck flask fitted with an efficient reflux condenser, a mechanical stirrer, and a tube for the introduction of nitrogen. This tube was arranged so that it could be partially withdrawn to permit introduction of the solid formylated dye. The flask was kept in a water bath held at 20° C. and a nitrogen atmosphere was maintained in the flask. After the hydride was suspended evenly 51 gm. (0.2 mole) of the N-formyl dye was introduced in 1- to 2-gm. portions during a 45-minute period. Care was taken that excessive boiling of the ether did not occur. After the dye addition the stirring was continued for 15 minutes. The excess hydride was destroyed by the dropwise addition of 50 ml. of ethyl acetate. The lithium aluminum-dye complex was decomposed by the addition of 50 ml. of ethyl acetate. The lithium aluminum-dye complex was decomposed by the addition of 100 ml. water, and the stirring was continued for another 10 minutes. One mole of 40 per cent sodium hydroxide solution was added, and the dye was extracted into ether with additions of small volumes of ethyl alcohol to control the formation of emulsions. The ether extracts were taken to dryness in vacuo, and the residue was dissolved in warm benzene. Three gm. of activated charcoal was added, and the suspension was passed through a short column of activated alumina. Dye remaining on the column was eluted with benzene. After removal of most of the solvent the dye solution was carefully applied to a large (5-cm. diameter, 50-cm. length) column of activated alumina. The chromatogram was developed with 1:1 benzene-hexane mixtures. Yield: 32 gm. (66 per cent, based on the N-formyl dye); m.p., 72° C. An analytical sample obtained by rechromatography of this dye, melted at 114°-115° C.

Anal.—Caled. for C_{15}H_{15}N_{3}O: N, 17.43. Found: N, 17.45.

2-Methoxy-4-monomethylaminoazobenzene (2-methoxy-MAB).—This compound was synthesized from 2-methoxy-AB by the formylation and reduction procedure described above for 3-methoxy-MAB. A 45 per cent yield of dye melting at 112°-114° C. was obtained. The analytical sample, prepared by rechromatography of this dye, melted at 114°-115° C.

Anal.—Caled. for C_{15}H_{15}N_{3}O: N, 17.42. Found: N, 17.43.

2-Methoxy-2-dimethylaminoazobenzene (2-methoxy-DAB).—The general coupling procedure previously described (10) was employed to prepare this dye from N,N-dimethyl-m-anisidine and diacetylated aniline. The tertiary amine was prepared by methylation of m-anisidine with methyl sulfate. Sixty per cent yields of 2-methoxy-DAB, m.p. 103°-105° C., were obtained. Rechromatography of the dye yielded an analytical sample which melted at 104°-105° C.

Anal.—Caled. for C_{16}H_{17}N_{3}O: N, 16.46. Found: N, 17.01.

2-Hydroxy-4-monomethylaminoazobenzene (2-hydroxy-MAB).—This compound was derived from 2-methoxy-MAB by the aluminum chloride O-dealkylation procedure described previously for the preparation of 3-hydroxy-DAB (13). Only 30-40 per cent yields of dye with m.p. 158°-160° could be obtained. The analytical sample had a m.p. of 159°-161° C.; upon analysis this sample gave satisfactory C and H values, but the N value was unaccountably low.

Anal.—Caled. for C_{15}H_{15}N_{3}O: C, 68.70; H, 5.76; N, 18.49. Found: C, 68.91; H, 5.75; N, 17.52.

Assay procedure.—Young adult male or female rats (Holtzman Rat Co., Madison, Wisconsin) with initial weights of about 200 gm. were housed in groups of four in screen-bottomed cages. Food and water were available ad libitum. The basal diet contained: vitamin-low casein (Nutritional Biochemical Corp.), 180 gm.; corn oil, 50 gm.; salts mixture (7), 40 gm.; glucose monohydrate (Cerelose, Corn Products Refining Co.), 729 gm.; choline chloride, 1 gm.; percomorphum liver oil (Mead), 300 mg.; calcium pantothenate, 7 mg.; thiamin hydrochloride, 3 mg.; pyridoxine hydrochloride, 2.5 mg.; and riboflavin, 2 mg. The dyes were dissolved in the corn oil with mild heat and were added to the diets at a concentration of 2.67 mmoles/kg (equivalent to 0.06 per cent of DAB). The methoxy dyes and 2-hydroxy-DAB were fed in the diet for 8 months; 2-hydroxy-MAB, avail-
able only in limited supply, was fed for 5 months. After these times the rats were maintained on the basal diet until they died or were killed because of poor condition or at the termination of the experiments at 9 or 11 months. Each of the animals was subjected to a routine gross examination which included the tissues of the abdominal and thoracic cavities, the mammary tissue, and the ear ducts. Each of the tumors was fixed in neutral 10 per cent formalin, sectioned at 6 µ, and stained with hematoxylin and eosin for histological examination. We are grateful to Dr. Henrik Hartmann of the Department of Pathology of this University for the histological diagnoses of these tissue specimens.

RESULTS
In agreement with much published data DAB induced malignant liver tumors in the majority of the male rats fed 0.06 per cent of this dye in the diet for 4 or 5 months (Table 1). No tumors were found at any other site in these rats. In contrast, only one malignant liver tumor was found in the rats fed 3-methoxy-AB, and none of the other seven dyes tested in these experiments induced any malignant liver tumors. Mild gross cirrhosis and an occasional regenerative nodule or hemorrhagic cyst were found in the livers of some of the rats fed 3-methoxy-AB. However, unlike the parent dyes, the 3-methoxy derivatives of AB, MAB, and DAB were distinctive in that each induced keratinizing squamous-cell carcinomas of the ear duct (Figs. 1 and 2) in 41–78 per cent of the rats which survived for at least 4 months. 3-Methoxy-AB and 3-methoxy-MAB appeared to be more active at this site than 3-methoxy-DAB; the two former compounds also induced adenocarcinomas of the small intestinal epithelium (Figs. 3 and 4).

| EXP. NO. | COMPOUND* | AV INITIAL WT. (Gm.) | AV WT. GAINED AT 3 WK. (Gm.) | TIME CURED (MO.) | SEX | SCR-VITAL AT 4 MO. | NO. RATS WITH MALIGNANT TUMORS OF THE:
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<td>20</td>
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<td>9/16</td>
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<td>5</td>
<td>M</td>
<td>15/16</td>
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** 3-MeO-AB | 235 | 25 | 8 | M | 11/13 | 0 | 0 | 0 0 0 | 0 | 0 0 | 0 | 0 |

| EXP. NO. | COMPOUND* | AV INITIAL WT. (Gm.) | AV WT. GAINED AT 3 WK. (Gm.) | TIME CURED (MO.) | SEX | SCR-VITAL AT 4 MO. | NO. RATS WITH MALIGNANT TUMORS OF THE:
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<td>5</td>
<td>M</td>
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* 2.67 mmoles/kg of diet.
† No. of rats alive at 4 mo./no. started on diet.
‡ These squamous-cell carcinomas, one on the lip and one on the skin of the back, were found at 11 mo.
§ One basal-cell carcinoma was found at 8 mo., and a squamous-cell carcinoma was found at 9 mo. Both were located on the skin of the back.
\( \frac{\text{a}}{\text{b}} \) A basal-cell carcinoma on the skin of the back was first observed at 11 mo.
** Three groups of four, four, and five rats were fed the basal diet described in "Methods," the basal diet plus 20 mg. of 3-methyl-1,4-naphthoquinone/kg, or the basal diet in which crude casein was substituted for vitamin-low casein for 5 weeks. This experiment was set up as a result of the loss of four of sixteen rats fed 3-methoxy-AB during the first 3 weeks of the first experiment; the objective was to find more favorable conditions for administration of the compound. Since there appeared to be no differences in the rats fed these three diets by 3 weeks, the animals from all three groups were fed the basal diet described in the "Methods" for the remainder of the experimental period.
†† These data were obtained at 9 mo., the time at which the surviving rats of Exp. 3 were killed.
in some rats. Five rats fed the 3-methoxy derivatives developed solitary tumors of the skin which measured up to 1 cm. in diameter and which on microscopic examination were classified as basal-cell or squamous-cell carcinomas of low malignancy (Figs. 5 and 6). Only 3-methoxy-AB was fed to female rats; three of the seventeen female rats which survived for at least 4 months developed adenocarcinomas of the mammary gland. However, one rat in the control group not fed any dye and one rat in the group fed AB also developed similar adenocarcinomas.

None of the rats fed the five remaining dyes, 2-methoxy-AB, 2-methoxy-MAB, 2-methoxy-DAB, 2-hydroxy-MAB, or 2-hydroxy-DAB, developed any gross tumors. However, it should be noted that the survival of the rats fed 2-methoxy-AB and 2-methoxy-MAB was poor for the first few months of the experiment. Some of these rats appeared to die as the result of hemorrhage as manifested by pericardial or abdominal blood clots, hemorrhagic testes, or bloody urine, but there was no other gross pathology.

DISCUSSION

One of the striking features of the carcinogenicity of DAB and related aminoazo dyes in the rat has been the predilection of these compounds to induce tumors in the liver (11). Tumor induction in extrahepatic sites in the rat by these compounds has been noted in only a few instances. Yoshida (25) induced sarcomas in rats by the subcutaneous injection of p-aminoazotoluene. Mulay and O'Gara (18) produced sarcomas in female rats with subcutaneous injections of N,N-dimethyl-p-(1-naphthyl)azoaniline. When the latter dye was fed to male rats it induced dermal-subcutaneous fibromas and adenofibromas, as well as papillomas and carcinomas of the forestomach (17). Two types of nonhepatic tumors have occurred in rats given DAB. These comprise primary pancreatic tumors in two of 30 rats fed DAB for 12–15 months in a diet which greatly inhibited liver tumor formation (5) and lymphosarcomas in the spleens of five of 28 rats fed and given injections intrasplenically of the dye for 15 months (6). The carcinomas induced in the ear duct and the small intestine by 3-methoxy-AB, 3-MAB, and 3-DAB represent the first cases of significant tumor induction at these sites in the rat by aminoazo dyes. Thus, tumor induction at these sites, particularly in the ear duct, appears to link the carcinogenic activity of the 3-methoxy dyes with that of aromatic amines such as 2-aminofluorene, 4-aminobiphenyl, 4-aminostilbene, benzidine, etc. (4, 9, 20–23). Most of these amines and their carcinogenic derivatives induce high incidences of tumors of epithelial origin at several sites in the rat which may include the liver, ear duct, small and large intestine, mammary gland, and kidney. Hence, the data on the 3-methoxy dyes provide some evidence that the carcinogenic aminoazo dyes belong to the general class of carcinogenic aromatic amines rather than to a special class of their own.

The reasons for the anomalous carcinogenic behavior of the 3-methoxy derivatives as compared with the other derivatives of AB, MAB, and DAB that have been tested are not clear. However, it may be relevant that the 3-methyl derivatives of MAB and AB are not reduced or ring-hydroxylated to an appreciable extent by rat liver homogenates (16). If, as seems likely, the 3-methoxy derivatives are similarly resistant to reduction and ring-hydroxylation, some fraction of these dyes may escape destruction by the liver and be available for reactions in other tissues. It is also possible that the 3-methoxy dyes are particularly prone to undergo benzidine rearrangements (12, 14) in vivo to yield derivatives of 4-aminobiphenyl with the ability to attack extrahepatic tissues. Likewise, the demonstration that the N-hydroxy metabolites of 2-acetylamino-4-fluorene and 4-acetylamino-biphenyl are proximate carcinogens in carcinogenesis with these amides in the rat (8, 24) raises the possibility that the 3-methoxy dyes are similarly metabolized to N-hydroxy derivatives which, for unknown reasons, are more active at peripheral sites than in the liver.

REFERENCES


Fig. 1.—Squamous-cell carcinoma in the ear duct. This section shows several keratin pearls in the process of formation and a large keratin-plugged follicle. X100.

Fig. 2.—Squamous-cell carcinoma in the ear duct. This section shows some cells in mitosis and the concentric arrangement of the cells which form the partially calcified pearl. X430.

Fig. 3.—Section of an adenocarcinoma of the small intestine which shows the development of the tumor from the normal glandular epithelium. X25.

Fig. 4.—Section of an adenocarcinoma of the small intestine which shows the irregular mucin-producing glands. X125.
Fig. 5.—Carcinoma of the skin. The epithelial down-growths are composed of uniform cells which are separated by connective tissue. ×100.

Fig. 6.—Carcinoma of the skin. In this tumor the epithelial cells are not keratinized and are, in part, oriented as basal cells. ×400.
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