The carcinogenic azo dyes normally give rise to tumors only in the liver when given to rats in the food; 3-methoxy-4-aminoazobenzene (3-methoxy-AB) and its N-mono-methyl and dimethyl analogs (3-methoxy-MAB and 3-methoxy-DAB) are therefore of particular interest, because they produce extrahepatic tumors (5).

Previous work in these laboratories has shown that, when 0.5 per cent cupric oxyacetate hexahydrate is included in a maize diet containing 0.09 per cent of the liver carcinogen 4-dimethylaminoazobenzene (DAB), liver tumors are rarely formed; whereas DAB alone gives rise to tumors in 100 per cent of our laboratory stock rats (4).

It has been suggested (1) that the protection may be concerned with competitive binding by the copper with the carcinogen for the available protein sites in the liver, and it was considered that experiments in which the copper salt was given together with the 3-methoxy dyes might be of value in elucidating the protective mechanism of copper by determining whether there was any effect on the induction of extrahepatic tumors.

This paper, therefore, presents the results from feeding experiments with the use of 3-methoxy-AB, 3-methoxy-MAB, and copper acetate.

MATERIALS AND METHODS

Chemicals.—3-Methoxy-AB (6) and 3-methoxy-MAB (5) were prepared as described earlier. The melting points agreed with those published.

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Cupric oxyacetate hexahydrate was obtained from Hopkin & Williams Ltd., Chadwell Heath, Essex, England—(CH₃CO₂)₂Cu·CuO·6H₂O containing 34.4 per cent copper.

Assay procedure.—Forty male albino 3- to 4-month-old rats of our outbred laboratory stock were used. Four diets were prepared: maize +0.09 per cent 3-methoxy-AB; maize +0.09 per cent 3-methoxy-AB +0.5 per cent copper acetate; maize +0.09 per cent 3-methoxy-MAB; and maize +0.09 per cent 3-methoxy-MAB +0.5 per cent copper acetate.

Each diet was given to ten rats for 5 days each week with proprietary rat food being given on Saturdays and Sundays. Full details of the method of preparing the maize-based diets, dietary regimens, and of housing the animals have been described previously (4).

The animals were killed with ether between 200 and 700 days after the start of the experiment, either because of ear-duct tumors or because of general ill health.

A full post-mortem examination was performed, and blocks of tissue were fixed in 4 per cent formaldehyde-saline. Sections were cut at 5 μ and stained with Ehrlich’s hematoxylin and eosin, and Weigert’s hematoxylin and Van Gieson.

The whole liver was removed from the animal, weighed, and whenever possible blocks were taken for histological examination. The remaining liver was washed with cold water, minced through a stainless steel mesh to retain connective and vascular tissue, and a sample of the resulting pulp was homogenized in saline. The homogenate (about 10 per cent) was assayed for nitrogen and copper by methods previously described (2).
RESULTS

General.—All the animals thrived on the experimental diets, and there were no early deaths. Daily food consumption rose from 10 gm. per rat initially to 25 gm. after 400 days' treatment and thereafter remained steady. There were no differences in food consumption among the four groups, and the amount consumed was the same as that when rats are given maize meal alone under similar conditions.

Experiments conducted previously (1) have shown that the ratio of body weight to liver weight affords a reliable index of the degree of tumor growth in rat liver. The mean value of this ratio for each dietary group is given in Table 1, together with the mean increase in body weight after an arbitrary period of 200 days.

The rats in the two groups receiving the N-methylated dye had to be killed an average of 4 months before those in the other two groups because ear-duct tumors developed more quickly. It is therefore possible that the lower weight ratio in Group 1 compared with Group 3 may be due to the shorter period of feeding rather than to differential activity of the two dyes. Certainly, the mean ratio for the first four rats killed in Group 1 (mean time, 336 days) was 18.8, which is almost identical with the Group 3 figure.

In both groups, adding the copper salt to the diet limited the decreases.

Tumors produced.—Tumors were produced in three sites—namely, ear duct, skin, and liver. The yield of tumors at these sites varied with the azo dye, but only in the case of the liver was there inhibition of the tumor development owing to copper feeding.

Ear duct tumors.—All twenty rats receiving 3-methoxy-MAB with or without copper produced these tumors, as did six out of ten rats given 3-methoxy-AB alone and seven out of ten rats given this dye plus copper acetate.

These tumors were rapidly growing, and animals bearing them usually had to be killed within 3–4 weeks of their first appearance. They apparently arose in Zymbal's gland, which at first showed severe squamous metaplasia followed later by frank invasive carcinoma, with the production of masses of keratin which quickly became secondarily infected. Invasion of adjacent bones was a prominent feature.

Skin tumors.—These were found only in rats given 3-methoxy-AB both with and without the addition of copper acetate. Thus, four rats of each group developed these tumors, and in one animal in each group they were multiple.

These tumors were first detected after an average of 341 days' treatment and were always situated on the dorsal skin surface. They were slowly growing, never exceeding 5 mm. in diameter, were sharply circumscribed and unattached to deeper structures. There was never any evidence of spontaneous regression.

Usually, on microscopic examination, these lesions situated within the dermis consisted of keratin-filled cysts lined by squamous epithelium. The epithelial lining was always regular, and there was never invasion of adjacent tissues. In other instances, the morphology of the skin tumors resembled that of a dome-shaped keratoacanthoma, consisting of a central plug of keratin surrounded by a rather irregular zone of squamous epithelium showing apparent infiltration (Fig. 1). Although early examples of these lesions were not seen, the appearances were consistent with an origin from the pilo-sebaceous apparatus.

Liver tumors.—All the rats given 3-methoxy-AB alone showed gross evidence of liver damage. Thus, in six animals the liver surface was irregular owing to the presence of regeneration nodules. In three animals there was gross evidence of a coarse multilobular cirrhosis. Cystadenomas were present in four livers, and in five there were large, solid masses of pure tumor replacing most of a lobe.

Histological examination of available material showed isolated necrotic liver cells scattered throughout the lobules in all cases. Fatty change in the parenchymal cells, especially in the perportal areas, was sometimes a conspicuous feature. Areas of liver cell regeneration were present in all specimens, and the nodules formed varied considerably in size, sometimes being intralobular and sometimes replacing several lobules (Fig. 2). It was noteworthy that the presence and degree of regeneration were disproportional to the fibrosis present, the latter frequently being minimal in amount. There were areas of focal dilatation and proliferation of bile ducts forming small cystadenomas which sometimes merged into one another, forming large multilobular cysts replacing large areas of liver tissue.

The large solid tumors were all well differentiated adenoid trabecular hepatomas composed of large cells, sometimes with multinucleated giant forms (Fig. 3).

When copper acetate was included in the diet the livers appeared normal on gross examination, with three exceptions—which showed slight granularity of the surface. However, microscopically there was no evidence of cirrhosis in these livers, but two showed small areas of regeneration and small groups of dilated bile ducts forming minute cystadenomas. Very occasional necrotic liver cells were present. The third liver presented an extraordinary microscopic appearance, having a spongelike structure due to enormous dilatation of the sinusoids unassociated with liver cell damage or other abnormality (Fig. 4).

Administration of the N-methylated dye had effects similar to those of 3-methoxy-AB on the liver. The length of the experiment was somewhat limited by the early development of ear duct tumors, but liver damage was similar in kind, although less severe in degree, than with the unsubstituted dye. Thus, moderate cirrhosis and regeneration nodules were seen in three animals, and cystadenomas were present in only two rats. Two animals had hepatomas, which again were of well differentiated, adenoid trabecular type composed of large cells together with occasional giant cells.

When copper was included in this diet, gross liver abnormalities were not detected, and also, on microscopic examination, the livers were normal apart from a slight accumulation of ceroid pigment.

Other lesions.—Two other tumors were encountered. One was a hemangioma of the mesenteric fat occurring in the rat given 3-methoxy-AB alone for 680 days. The other lesion was a pleomorphic spindle-cell sarcoma oc-
TABLE 1

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Compounds</th>
<th>AV. period of treatment (days)</th>
<th>AV. wt. gain at 200 days (gm.)</th>
<th>POST-MORTEM RATIO OF BODY WEIGHT TO LIVER WEIGHT</th>
<th>INCIDENCE OF TUMORS OF THE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-MeO-AB</td>
<td>463 (266-680)</td>
<td>193</td>
<td>13.6</td>
<td>5 6 4</td>
</tr>
<tr>
<td>2</td>
<td>3-MeO-AB + CuAc</td>
<td>452 (231-641)</td>
<td>219</td>
<td>21.3</td>
<td>0 7 4</td>
</tr>
<tr>
<td>3</td>
<td>3-MeO-MAB</td>
<td>327 (200-423)</td>
<td>208</td>
<td>19.5</td>
<td>2 10 0</td>
</tr>
<tr>
<td>4</td>
<td>3-MeO-MAB + CuAc</td>
<td>348 (228-420)</td>
<td>194</td>
<td>24.8</td>
<td>0 10 0</td>
</tr>
</tbody>
</table>

* Ten male rats were used in each experiment.
† Normal (maize-fed) value 26.6 after comparable feeding time.
↑ These values represent the number of animals developing the various tumors. The actual number of tumors was higher than those values in the cases of liver and skin where multiple tumors were often found. Ear duct tumors, however, were always unilateral.
§ Both dyes given at 0.09 per cent; MeO = methoxy.
# Cupric oxyacetate hexahydrate, 0.5 per cent.

The yield of tumors is summarized in Table 1.

*DISCUSSION*

The incidence of ear duct and skin tumors is similar to that reported by Miller and Miller (5), except that the skin tumors in their experiments were considered to be malignant. The present results differ in that the Millers described a small incidence of liver damage (one malignant tumor with 3-methoxy-AB not classified histologically; mild cirrhosis and an occasional regenerative nodule) and several adenocarcinomas of the small intestine, whereas our animals displayed a higher degree of liver damage but no intestinal tumors, despite careful search. The Millers terminated their experiments after 11 months, and it is possible that their animals would have produced liver tumors had the period of treatment been extended. An alternative explanation is that in our rats sufficient dye was metabolized to the active hepatocarcinogen(s), whereas this did not occur in their Holtzman rats.

When the histological changes in the liver caused by the administration of the 3-methoxy dyes are compared with those produced by DAB, certain differences can be noted. The former apparently cause no great increase in perportal cellularity, with the development of chronic inflammatory granulation tissue which is such a prominent feature of DAB carcinogenesis. Furthermore, the development of cirrhosis is not so marked with the 3-methoxy dyes as with DAB, whereas areas of liver cell regeneration appear more marked than with DAB. The extent of regeneration appeared out of proportion to the amount of cirrhosis. It is of interest that areas of cholangiofibrosis were never observed, either grossly or microscopically, in the animals fed either 3-methoxy dye. Similarly, there were no examples of cholangiocarcinoma, the neoplasms present being pure hepatomas. Mixtures of cholangiocarcinoma and hepatoma such as are so frequently seen in DAB-induced hepatic tumors were not observed.

Tumor induction time in the liver was much greater with the methoxy dyes than with DAB, where the average induction time is 8.5 months (4). This would be expected if only a proportion of the dye molecules are metabolized to hepatocarcinogen; but the fact that tumors are eventually produced indicates that an unsubstituted 3-position is not essential for carcinogenesis, either because this position is not involved in the process or because the methoxy group is easily removable in vivo.

A reaction to DAB feeding in our rats is an increase in currying in the leg muscles of a rat given the same dye with copper acetate for 398 days.

The whole liver copper content in animals fed either dye alone, expressed as µg/mg protein nitrogen, was within the normal range, in contrast with previous results when DAB was used (1-3), in which case increased copper storage was found during carcinogenesis.

When the copper salt was also given there was an enormous storage to mean values of 67 times normal with 3-methoxy-AB and to 36 times normal with the N-methylated dye. Protein samples were prepared from the liver homogenate of the last rat killed in each of these two experiments, extracted exhaustively with 95 per cent ethanol in a Soxhlet apparatus and assayed for copper. In both cases values of 45 µg protein-bound copper per gm. of liver pulp were obtained, in good agreement with the 'saturation' value of about 40 µg/gm of wet liver found previously after long-term copper administration with DAB (1).
copper storage in the liver of 40 per cent after a year's treatment (1-3). This increase is purely a function of the time of treatment, since unpublished experiments have shown that the increase occurs irrespective of the tumor induction time when this is varied by stepping up the concentration of riboflavin in the diet. The extra copper is attained at the expense of a corresponding decrease in the kidneys (3), and the effect was noted before the development of tumors. No such increase was observed in these experiments, and it is felt that this fact may be relevant to the different sites of tumor development in these animals.

It is suggested that the protection by copper feeding is due to competitive binding with the carcinogen for the available protein sites in the liver as suggested for DAB (1). In these experiments, copper determinations were occasionally made of skin and ear duct tissue, both normal and tumorous, from rats given copper-containing diets. No evidence of increased copper storage was noted in these tissues, and there appeared to be no protection given against tissue damage by the dyes.

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