

# The Production of Tumors in the Livers of Rats Fed *m*'-Methyl-*p*-Dimethylaminoazobenzene\*

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(Received for publication October 25, 1946)

Miller and Baumann (3) and Giese, Miller and Baumann (1) studied the carcinogenicity of various methyl derivatives of dimethylaminoazobenzene and found that the *m*'-methyl-*p*-dimethylaminoazobenzene was the most potent of the azo dyes for the production of liver tumors in rats, being even more active than *p*-dimethylaminoazobenzene itself. As we were interested in studying conditions of rapid carcinogenesis, *m*'-methyl-*p*-dimethylaminoazobenzene was selected as the carcinogenic agent.

In most of the early studies on the production of liver tumors, the investigators fed the dye continuously to the rats until the tumors appeared. Kinoshita (2) mentioned in one of his reports that if animals were fed *p*-dimethylaminoazobenzene in the rice-carrot diet for only 50 days, and then returned to a normal diet without the dye, some of the rats would develop tumors in 150 days. Sugiura and Rhoads (9) fed *p*-dimethylaminoazobenzene to rats for 30, 45, 60, and 85 days in the rice-carrot diet, and then withdrew the dye from the diet, continuing the animals on the rice-carrot diet alone. Only those animals which received the dye for 60 and 85 days later developed tumors. Reimann, Stimson, and Medes (8) fed rats *p*-dimethylaminoazobenzene in a rice diet supplemented with fresh vegetables for 75 days and then returned them to the stock diet. One hundred and thirty days later they found that 5 out of 15 animals had developed tumors. Giese, Miller and Baumann (1) fed the *m*'-methyl-*p*-dimethylaminoazobenzene at a level of 0.056 per cent in a semi-synthetic diet for 2½ months and then continued the animals on the semi-synthetic diet alone. Two months later there was a 100 per cent incidence of liver tumors.

In the studies reported here, the animals were fed the azo dye for different lengths of time in an effort to determine the minimum possible exposure to the

carcinogenic agent necessary for subsequent tumor formation. In addition, some information was obtained on the rôle of diet in the development of tumors following the initial period of dye feeding. In the early studies on the production of liver tumors with the azo dyes both the Japanese workers and the investigators in this country fed the dye to rats in a diet consisting of rice and carrots. The use of this diet, however, carried with it a relatively high mortality, as a rule. Miller, Miner, Rusch, and Baumann (4) and Miner, Miller, Baumann, and Rusch (5) in careful studies on the relation of diet to hepatic tumor formation found that a semi-synthetic diet low in protein and riboflavin but more than adequate in respect to other members of the vitamin B complex resulted in a very high incidence of tumors when the azo dye was incorporated in it, and at the same time, provided a diet adequate for maintenance of the animals. They found that increasing the riboflavin content of such a diet could prevent tumor formation completely. With the use of more crude diets, increase in the protein content of the diet reduced considerably the incidence of tumor formation. In the experiments reported below, the rats were fed the *m*'-methyl-*p*-dimethylaminoazobenzene in the semi-synthetic diet as described by Miller, Miner, Rusch, and Baumann (4), but following the initial period of dye-feeding some of the animals were placed on the regular stock diet and the subsequent course of tumor development was followed.

## METHODS

A total of 91 adult male rats of the Sprague-Dawley strain weighing between 230 to 320 gm. at the start of the experiment were used. All of the rats were placed at first on the semi-synthetic diet of Miller, Miner, Rusch and Baumann (4), consisting of crude casein, 120 gm.; salts, 40 gm.; corn oil, 50 gm.; rice bran concentrate, 20 gm.; glucose, 770 gm.; and riboflavin, 0.5 mgm. per kgm. of diet. In addition each rat was given 1 drop of halibut liver oil monthly. Into this diet was incorporated 0.05 per cent of *m*'-methyl-

\* Aided by grants from The Donner Foundation and The Jane Coffin Childs Memorial Fund for Medical Research.

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*p*-dimethylaminoazobenzene by dissolving it by means of heat in the corn oil. The dye was synthesized according to the method described by Giese, Miller and Baumann (1). After the animals were on this diet for approximately 1 month, they began to show signs of riboflavin deficiency. The rice bran concentrate was increased to 30 gm. and the riboflavin to 0.75 mgm./kgm. of diet, after which all signs of riboflavin deficiency disappeared.

At various intervals after beginning the dye feeding, the exact times of which are stated below, a group of animals were removed from this dietary regime. Half of such a group was then placed on fox chow (Purina) and the other half was maintained on the semi-synthetic diet described above, but without the dye. The animals were then observed until tumors developed, or for approximately 190 days, at which time all remaining animals were killed. The abdomen was palpated for the presence of a tumor and the first appearance of such was noted. Usually animals were not killed until the tumors had grown to considerable size, but in several cases rats died as the result of the tumor growth. At various intervals in the experiment representative rats were killed for examination of the liver both grossly and microscopically.

All the rats were allowed food and water *ad libitum*. Food intake was not measured but body weight was recorded weekly to determine the progress of the animals.

### RESULTS

Eleven rats received *m'*-methyl-*p*-dimethylaminoazobenzene in the semi-synthetic diet for 39 days. Following this 5 of the rats were continued on the semi-synthetic diet without the dye and 6 were given fox chow. These rats were followed for an additional 146 days, at which time they were sacrificed and their livers examined for the presence of tumors. In none of these animals was there any evidence of tumor formation, either grossly or microscopically.

Fourteen rats received the *m'*-methyl dye in the semi-synthetic diet for 46 days. Then 7 rats were given fox chow and 7 were continued on the semi-synthetic diet without the dye. These rats were followed for an additional 151 days, at which time they were sacrificed. In this group likewise there was no evidence of any tumor formation.

Thirty rats received the *m'*-methyl dye for 69 days. Following this the rats were divided into two groups; 15 rats were continued on the semi-synthetic diet without the dye, and 15 rats were fed fox chow for the duration of the experiment. The first palpable tumor appeared in a rat receiving the semi-synthetic diet on the 24th day following discontinuance of the dye feeding. This was slow-growing and did not

become of any considerable size until 90 days after discontinuance of the dye. In the group receiving fox chow, the first tumors appeared on the 42nd and 55th days after discontinuance of dye feeding. On the 81st day following discontinuance of dye feeding, of the 15 rats in the fox chow group, 10 already showed evidence of tumor formation, either by the definite presence of a palpable mass in the abdomen or demonstrated by autopsy. In the group receiving the semi-synthetic diet alone, 6 of 15 showed evidence of tumor formation by the 81st day. On the 115th day after discontinuance of the dye feeding, all remaining animals that had not died or been sacrificed in the meantime were killed and examined for tumor formation. Final count in the two groups showed the following tumor incidence: 14 of 15 animals on fox chow had tumors, an incidence of 93 per cent, whereas 12 of 15 animals on the semi-synthetic diet alone had tumors, an incidence of 80 per cent. The data on the relation of the duration of dye feeding to the incidence of tumor formation are summarized in Table I.

TABLE I: RELATION OF THE DURATION OF DYE FEEDING, AND OF THE SUBSEQUENT DIET TO THE PRODUCTION OF LIVER TUMORS IN RATS FED *m'*-METHYL-*p*-DIMETHYLAMINOAZOBENZENE

No. of rats	No. of days on exper. diet plus azo dye	No. of days on exper. diet alone	No. of days on fox chow alone	No. of rats with tumors 81 days after dye stopped	No. of rats with tumors, final incidence
6	39		146	0	0
5	39	146		0	0
7	46		151	0	0
7	46	151		0	0
15	69		81-115	10	14
15	69	81-115		6	12

In the course of the autopsy of these rats the lungs were examined grossly for any suspicious nodules, and if such were present, microscopic examination of the nodules were carried out. Since some of the animals died as a result of their tumors, only those animals on which adequate autopsy was performed are reported here. Of the 7 rats receiving the semi-synthetic diet alone, the lungs of which were examined carefully following death, none showed any gross evidence of the presence of tumor nodules of any kind. Of the 9 rats on the fox chow diet, which were similarly autopsied, 5 showed definite gross and microscopic evidence of the presence of tumor metastases varying in size from 1 mm. to as large as 8 mm. These were in all cases identified as coming from the liver tumors. Although no microscopic examination was made of the lungs of animals showing no nodules grossly, still it is possible to conclude from the data presented here that at least the extent of tumor metastases was significantly greater in rats whose diet following the period of dye ingestion was fox chow.

The 30 rats that ingested the azo dye for 69 days showed the following weight changes. During the course of the dye feeding, the average gain per rat for the entire group over the 69 day period was 7 gms. For the 15 rats on fox chow, the average gain per rat after 81 days was 104 gm. The rats placed on the semi-synthetic diet alone for the corresponding period showed an average increase of 45 gms. per rat. Incorporation of the azo dye into the semi-synthetic diet prevented almost completely any weight increase which the animals would have had on the semi-synthetic diet alone. Although food intake was not measured, the much greater increase in weight of the animals that were continued on fox chow indicates a food consumption greater than that in animals maintained on the semi-synthetic diet alone.

Microscopic examination of the tumors appearing in the liver in the course of these experiments showed that in general the types of tumors produced and their development following feeding with *m'*-methyl-*p*-dimethylaminoazobenzene were very similar to those produced in rats by *p*-dimethylaminoazobenzene, as described by Orr (7) and Opic (6). Like these authors we found that the tumors could be classified as bile duct carcinoma (cholangioma), bile duct cystadenoma and liver cell carcinoma, and that in many instances more than one type of tumor appeared in the same animal.

#### DISCUSSION

These studies have shown that in the production of tumors in rat livers by feeding *m'*-methyl-*p*-dimethylaminoazobenzene in a semi-synthetic diet low in protein and riboflavin, the duration of feeding the dye will determine the rate of tumor formation. Although it is not necessary to administer the carcinogenic agent continually, nevertheless the animals must be exposed to its effects for a minimum length of time in order that tumors may develop. In animals that ingested the dye for only 39 and 45 days tumors never developed, whereas animals exposed to the dye for 69 days later developed a high incidence of tumors. It would seem that during this period the neoplastic focus has developed which can now proceed independently of the exciting agent to the formation of tumor.

In liver carcinogenesis with the azo dyes, the simultaneous feeding of an inadequate diet, particularly in respect to protein and riboflavin, has been found necessary for the production of a high incidence of tumors. These experiments show, however, that once the neoplastic focus has arisen, the maintenance of this low-protein, low-riboflavin diet is not essential for the further growth and development of the tumors. The animals receiving the azo dye in the semi-synthetic diet for 69 days and then receiving the fox chow diet

showed a high incidence of tumor formation. In comparing these animals with those maintained on the semi-synthetic diet alone, the final incidence of tumor formation on the fox chow diet is slightly greater (14 of 15 compared with 12 of 15) but not significantly so because of the relatively small number of animals used in the series. However, the animals receiving the fox chow diet after their initial exposure to the azo dye showed an earlier onset of tumor formation as indicated by the greater tumor incidence at 81 days as compared with the animals on the semi-synthetic diet. Furthermore the tumors of the group fed fox chow showed a significantly greater development of lung metastases.

Tannenbaum (10) has clearly shown for many different types of mouse tumors, both spontaneous and induced, that restriction of caloric intake decreases the incidence and delays the formation of tumors. The greater weight gain of the animals fed fox chow indicates a higher food consumption for these animals. Their increased caloric intake undoubtedly accounts in great part for the earlier development of hepatic tumors and their more widespread metastases as compared with the animals on the semi-synthetic diet, whose food intake was lower. Evidence for the role of the other dietary factors, such as changes in protein and riboflavin level, on tumor development following the minimum period of exposure to the azo dye cannot be adduced from the data presented here because of the co-existing variations in caloric intake, which of themselves influence tumor development.

#### SUMMARY

1. Rats fed *m'*-methyl-*p*-dimethylaminoazobenzene in a semi-synthetic diet low in protein and riboflavin for 39 and 46 days, then followed for 150 days without the dye, developed no liver tumors whether the diet during the dye-free period was semi-synthetic or fox chow.

2. Rats which were fed the azo dye in the semi-synthetic diet for 69 days, and then placed on dye-free diet, developed a high incidence of tumors. Approximately 93 per cent of the animals placed on fox chow following the dye-feeding period had tumors, whereas 80 per cent of those maintained on the semi-synthetic diet developed tumors. The tumors of the animals receiving fox chow appeared earlier and were more malignant as evidenced by the greater incidence of lung metastases.

3. The histopathology of liver tumors produced by *m'*-methyl-*p*-dimethylaminoazobenzene was found to be similar to that of tumors produced by the parent dye, *p*-dimethylaminoazobenzene, namely liver cell carcinoma, bile duct cystadenoma, and cholangioma.

## ACKNOWLEDGMENT

I am grateful to Dr. H. S. N. Greene, of the Department of Pathology, who reviewed some of the tumor slides with me.

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