

Some Dietary Factors in Experimental Liver Cancer

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Introduction

Feeding azo dyes in purified diets leads to development of lesions and eventually to liver cancer. Several derivatives of 4-dimethylaminoazobenzene have been tested for comparative carcinogenicities and a most effective compound was found to be 3'-methyl-4-dimethylaminoazobenzene (1).

Most dietary formulas used in azo dye carcinogenesis during the past twenty years involved addition of the dyes to semi-synthetic diets containing highly processed ingredients coupled with low quantities of riboflavin (1, 2, 3, 4, 5). It has been reported that diets deficient in riboflavin were conducive to tumor development, whereas tumors developed more slowly, or failed to appear in the experimental period, when dietary levels of riboflavin were increased (1, 5). That analogs of riboflavin inhibit flavoenzymes or influence riboflavin metabolism in a variety of biological systems (6) provides reasoning along the lines that addition of such analogs to diets containing azo dyes would modify the course of carcinogenesis and perhaps accelerate the carcinogenic process.

The primary purpose of the experiments reported here was to determine whether 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) would retain its carcinogenicity in rats fed a non-processed stock diet adequate in all dietary components, including riboflavin. Also, since 6, 7-dimethyl-9-(2'-acetoxyethyl)-isoalloxazine (U-2112) was shown to be an effective riboflavin antagonist (7) another purpose of these studies was to determine whether this analog could change the susceptibility of rats to the carcinogenic influence of 3'-Me-DAB.

Materials and Methods

Mature rats of the Wistar strain were fed a powdered commercial diet prepared from pellets produced by Pilsbury's Ltd. The dietary ingredients were natural products consisting primarily of whole grains, dried milk, yeast, molasses, salts, cod liver oil; and meals of meat, bone, and fish. On Friday of each week, the dried food was withdrawn and the rats given fresh greens.

Mature rats were divided into three groups and treated as follows: group 1 consisted of 6 male and 6 female controls fed stock diet to which no chemical agents were added; group 2 consisted of 5 males and 6 females fed azo dye mixed in the stock diet; group 3 consisted of 5 males and 5 females fed stock diet containing azo dye and riboflavin antagonist (U-2112). The dye (3'-Me-DAB) comprised 0.06% of the diet for groups 2 and 3 and the riboflavin antagonist was mixed into the diet in a con-

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centration of 20 mg/kg diet and fed to rats of group 3 for 3 weeks; the concentration was raised to 1 gm/kg and fed for another 5 weeks.

Animals were maintained on dietary regimes for periods varying from 125 to 131 days or approximately 4 months. Rats in group 3 received riboflavin antagonist during the first two months only but they were given 3'-Me-DAB for a 4 month period.

During the course of the experiments, rats were checked daily and weighed periodically. At the termination of the experiment the rats were weighed, sacrificed and bled from the juglar vein. The livers were weighed, examined macroscopically for lesions and portions of the liver were removed for microscopic study.

Results

As can be seen in the tables, both male and female rats on the stock diet (group 1) gained appreciably in body weight over the 4 month experimental period, thus indicating the adequacy of the stock diet. Livers of these animals were normal in all respects.

Males maintained on the diet containing 3'-Me-DAB (group 2) gained less weight than controls, whereas females lost weight on this regime. On a body weight basis the livers of both males and females were slightly larger than those of controls. Livers from all males had easily detectable lesions and microscopic examination of all such lesions revealed adenocarcinomas. Lesions were present in 4 of 6 livers from females but such lesions contained no cancers. These lesions were probably of a precancerous nature; histologically they consisted of hypertrophied, inflamed, and degenerate liver cells.

In group 3, the males fed azo dye and riboflavin analog in the diet gained as much weight as control rats, and females on the same regime showed a weight gain, but somewhat less than that observed in controls. Livers from either sex showed no signs of enlargement, being about the same weight as livers from controls. In examining the livers in this group, 4 of 5 livers from males showed lesions but none of the female livers appeared abnormal. Lesions in the male livers consisted of whitish blotchy areas on the surface with little indication of nodule formation. They were less distinctive than lesions observed in livers from males of group 2 (azo dye but no riboflavin analog). Although none of the livers in this group exhibited carcinomas there were histological signs of degeneration in most of the livers of both sexes. Cellular hypertrophy was lacking, but increased in connective tissue and fatty infiltration was obvious in the livers from 3 males and 4 females.

Discussion

Results of these experiments show clearly that azo dye carcinogenesis will take place in male rats fed a stock diet containing 3'-methyl-4-dimethylaminoazobenzene. Liver enlargement, after 4 months of azo dye feeding, is not as great as that seen when the dye is fed in a semi-synthetic diet low in riboflavin (8) and the degree of neoplastic involvement is less. Tumor development took place more slowly in animals fed stock diets and azo dye, but extension of the experimental period probably would have resulted in increased size and number of carcinomatous areas.

TABLE 1. Mean body weights of rats fed various diets for four months.

Group	Diet	Sex	No.	Initial B.W. g	Final B.W. g	B.W. change g	Liver wgt. g
1	Stock	Male	6	340	443	+103	15.2
2	Stock + azo dye	Male	5	262	328	+ 66	13.6
3	Stock + azo dye + riboflavin analog	Male	5	188	303	+115	9.1
1	Stock	Female	6	330	419	+ 89	13.6
2	Stock + azo dye	Female	6	290	276	— 20	9.7
3	Stock + azo dye + riboflavin analog	Female	5	224	267	+ 43	8.3

TABLE 2. Macroscopic and microscopic changes in livers of rats fed azo dye for four months.

Group	Diet	Sex	No.	Liver wgt. g/100g B.W.	No. with lesions	No. with carcinomas
1	Stock	Male	6	3.5	0	0
2	Stock + azo dye	Male	5	4.1	5	4
3	Stock + azo dye + riboflavin analog	Male	5	3.1	4	0
1	Stock	Female	6	3.1	0	0
2	Stock + azo dye	Female	6	3.6	4	0
3	Stock + azo dye + riboflavin analog	Female	5	3.1	0	0

The finding that most females on this dietary regime showed lesions but factor, especially in view of the fact that females lost weight during the experimental period, is that they voluntarily reduced food intake and hence consumed less azo dye than males. Difference in susceptibility of the two sexes to azo dye carcinogenesis has been reported by others (9), and on the basis of their findings it appears likely that had the females been allowed to continue eating the diet for a longer period of time they would have eventually developed adenocarcinomas. This point could be readily verified by further experimentation.

Addition of riboflavin analog (6, 7, dimethyl-9 (2'-acetoxyethyl)-isoalloxazine) to the diet did not enhance the carcinogenicity of 3'-Me-DAB. It is possible that this analog did not act, under these conditions, as a riboflavin antagonist because it is fairly well established that riboflavin deficiency increases the carcinogenicity of azo dyes (1, 5). It is just as reasonable to assume that it acted as a supplement to riboflavin, since all animals receiving the analog had normal size livers free of tumors. Also, livers of females in this group were free of lesions, and livers from males deviated from normal to a much less extent than those in group 2 where 3'-Me-DAB alone was added to the diet. Thus, it would appear that 6, 7, dimethyl-9 (2'-acetoxyethyl)-isoalloxazine exerted an anticarcinogenic effect under the conditions of these experiments. The mechanism of how it could act as an antagonist to 3'-Me-DAB remains to be elucidated, but it could be involved in the synthesis of enzyme systems that degrade azo dye. Regardless of such considerations, its apparent anticarcinogenicity here is in line with the findings of others who reported it to cause regression of established tumors in rats (7).

Summary

These studies showed that azo dye carcinogenesis can occur in rats fed a complete diet containing 3'-methyl-4-dimethylaminoazobenzene. Males were more susceptible than females. It probably would require a longer experimental period to get massive tumors with this regime compared to the procedure where azo dye is added to a semisynthetic diet low in riboflavin.

Addition of the riboflavin analog 6, 7, dimethyl-9- (2'-acetoxyethyl)-isoalloxazine to a stock diet containing 3'-Me-DAB did not enhance azo dye carcinogenesis but apparently inhibited such a process. Thus it would appear that this riboflavin analog was anticarcinogenic under the condition of these experiments. It would be interesting to test other riboflavin analogs and antagonists for their anticarcinogenicity in azo dye feeding.

Literature Cited

1. MILLER, J. A., R. W. SAPP, and E. C. MILLER. 1949. Carcinogenic activities of certain halogen derivatives of 4-dimethylaminoazobenzene in the rat. *Cancer Res.* **9**: 652-660.
2. DAVANZO, JOHN P. and W. J. EVERSOLE. 1958. The effects of adrenalectomy and desoxycorticosterones on liver enzymes and protein in rats fed 3'-methyl-4-dimethylaminoazobenzene. *Cancer Res.* **18**: 796-801.
3. EVERSOLE, W. J. 1958. The role of the adrenal cortex in azo dye carcinogenesis. *Lavori Dell Istituto di Anatomia E Istologia Patologica Della Università Degli Studi di Perugia.* **18**: 25-36.

4. ROBERTSON, C. H., M. A. O'NEAL, A. C. GRIFFIN, and H. L. RICHARDSON. 1953. Pituitary and adrenal factors involved in azo dye liver carcinogenesis. *Cancer Res.* **13**: 776-779.
5. KENSLE, C. J., K. SUGIURA, N. F. YOUNG, C. R. HALTER, and C. P. RHOADS. 1941. Partial protection of rats by riboflavin with casein against liver cancer caused by dimethylaminoazobenzene. *Science.* **93**: 308-310.
6. FALL, H. H., and H. G. PETERING. 1956. Metabolite inhibitors. I. 6,7-dimethyl-9-formylmethylisoalloxazine, 6,7-dimethyl-9- (2'-hydroxyethyl)-isoalloxazine and derivatives. *J. Am. Chem. Soc.* **78**: 377-380.
7. LANE, M., J. L. FAHEY, R. D. SULLIVAN, and G. G. ZUBROD. 1958. The comparative pharmacology in man and the rat of the riboflavin analogue, 6,7-dimethyl-9-(2'-acetoxyethyl)-isoalloxazine, U-2112. *J. Pharm. and Exper. Therap.* **122**: 315-326.
8. EVERSOLE, W. J. 1961. Adrenal cortical accessory tissue and azo dye carcinogenesis. *Proc. Ind. Acad. Sci.* **71**: 374-377.
9. RUMSFELD, H. W., JR., W. L. MILLER, JR., and C. A. BAUMANN. 1951. A sex difference in the development of liver tumors in rats fed 3'-methyl-4-dimethylaminoazobenzene or 4'-fluoro-4-dimethylaminoazobenzene. *Cancer Res.* **11**: 814-819.