The Effect of Riboflavin on the Liver Changes Produced in Rats by p-Dimethylaminoazobenzene

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(Received for publication June 6, 1942)

As part of a study on the relation of nutrition to the production of tumors by carcinogenic agents, *p*-dimethylaminoazobenzene was used as a tumor-producing substance in rats maintained on synthetic diets to which individual vitamins were added in known amounts.

It was found by Kinosita (4) that, among other dyestuffs, *p*-dimethylaminoazobenzene produced cirrhosis and carcinoma when fed to rats. Rats maintained on a diet of polished rice were less resistant to the action of this compound than those maintained on unpolished rice. The influence of vitamins in these diets upon the activity of the dye was studied by other Japanese workers (6, 7), who found that the addition of yeast or liver to the rice diet prevented the formation of tumors. Sugiura and Rhoads (8) obtained analogous results when yeast (15 per cent), yeast extracts, and rice bran extracts were incorporated in the diet. The addition to the rice diets of thiamin, pyridoxine, nicotinic acid, or liver eluate, each substance alone or in combination with others, did not prevent the development of tumors (7).

The relationship between susceptibility to tumor development and the inclusion of vitamins of the B complex in the diet was further stressed by Rhoads and his collaborators, who demonstrated a notable increase in the riboflavin excretion of rats maintained on a diet of brown Texas rice supplemented with 0.06 per cent *p*-dimethylaminoazobenzene (2), and who showed that the livers of such rats had a diminished content of both riboflavin and coenzyme I (3). These investigators have also proved that the addition of both casein and riboflavin is necessary to prevent liver damage in rats maintained on the rice diet and that neither of these substances is effectual when administered alone (2). White (9) observed that rats kept on a low protein diet containing *p*-dimethylaminoazobenzene failed to gain weight normally, and that the addition of cystine or methionine prevented the retardation in growth caused by the carcinogen. György and his associates (1) reported that casein, or the combination of methionine, cystine, and choline, protected rats against pathological changes in the liver produced by various diets containing *p*-dimethylaminoazobenzene. Lewisohn's group (5), studying the prevention of growth of transplanted carcinoma in mice, found that the inhibiting effect of yeast was definitely improved by the addition of pantothenic acid or riboflavin.

As a result of the suggestion contained in the experiments of Rhoads, the effect of riboflavin and nicotinic acid on the tumor-producing action of *p*-dimethylaminoazobenzene when added to synthetic diets has been investigated in albino rats originating from a Wistar strain (total number, 78). The animals, housed in individual cages, were placed at 3 weeks of age on a riboflavin-free synthetic diet consisting of vitamin-free casein, 18 per cent; dextrose, 68 per cent; butter fat, 8 per cent; salt mixture U.S.P. XI No. 1, 4 per cent; and cod liver oil, 2 per cent. Of this diet 1 kg. was supplemented with 8 mgm. each of thiamin and pyridoxine, 50 mgm. of calcium pantothenate, and 1 gm. of choline chloride. *p*-Dimethylaminoazobenzene, in an amount of 0.02 per cent, was incorporated in the food either at the beginning of the dietary regimen (48 rats), or after a period of 3 weeks during which the rats maintained on the riboflavin-free diet received 10 γ of riboflavin 3 times a week (30 rats). Forty-four animals were given no further supplement of riboflavin from the beginning of the feeding of *p*-dimethylaminoazobenzene, whereas the other 34 received 10 mgm. of riboflavin 3 times a week by stomach tube. The riboflavin was administered either in an aqueous suspension or suspended in 0.5 cc. of gum acacia. Twenty-four animals received a supplement of nicotinic acid (50 mgm. 3 times weekly). Control groups of animals receiving no *p*-dimethylaminoazobenzene in the diet (total number, 30) were kept simultaneously over the same period of time. To another group, given *p*-dimethylaminoazobenzene in the diet, was administered gum acacia alone.

After 6 weeks on the riboflavin-free diet, the weights of the rats fed the dye became almost stationary at 60 to 80 gm. and generalized alopecia developed. No significant difference in weight or in external appearance was found between these rats and the control rats receiving no *p*-dimethylaminoazobenzene. The majority of the animals died within 6 to 8 weeks; only one-third survived for periods of more than 2 months. Rats that had the supplement of nicotinic acid succumbed somewhat earlier than those given no nicotinic acid.

All rats fed 10 mgm. of riboflavin 3 times a week continued to gain weight throughout the experiment and presented a normal healthy appearance. After 5 months these animals averaged 350 gm. in body weight and their weight curves did not differ significantly from those of control rats receiving no *p*-dimethyl-

aminoazobenzene. In a group of 4 rats, the riboflavin supplement was withdrawn after a period of 160 days. These rats responded with a gradual decline in weight and died within 5 to 6 months. All the animals were examined at intervals, as recorded in Table I, summarizing the liver changes noted.

The livers graded "o" showed no definite gross or histologic changes. This does not signify that minimal changes had not taken place. For example a rare nucleus might be larger or stain more deeply, or a cell might contain 2 nuclei, but on the whole the cell type was uniform and at most the lesions were only suggestive of those graded ±.

The livers graded ± showed minor changes in comparison with the higher grade. Some of these consisted of occasional cells with a more eosinophilic cytoplasm or larger nucleus. In most instances, the changes occurred in isolated cells rather than groups of cells. When they did occur in cell groups, the foci were considerably smaller and the changes less advanced than those found in livers graded 1+. A rare area of necrosis was found in some of the ± livers.

In the livers graded 1+ there were foci with dilated capillaries and smaller liver cells. The cytoplasm of these cells was usually pale, less eosinophilic, at times vacuolated, and the cells were often multinucleated. These foci were usually nearer the center of the lobule. The cells in the periportal region also were smaller, but in these the cytoplasm stained more densely; occasionally an increase in the number of bile ducts was seen. A rare hyaline thrombus was found in a capillary and occasionally a macrophage containing brown pigment was noted.

The livers graded 2+ showed varying degrees of disorganization. Extremely cellular areas, often nodular, were present, composed of smaller cells often running parallel to one another. These resembled the bile duct cells because of their scant cytoplasm, but bile duct formation by these cells was not striking. Some of the cells contained yellow pigment. Many of these cells were elongated, simulating fibroblasts and endothelial cells, and at times were stellate. These cellular areas appeared to encroach upon the adjacent liver tissue. Scattered cells with large nuclei, many of which had more than one nucleolus, were seen throughout. These were not in any fixed relation to the periportal fields but were irregularly situated, and had an abundant, often eosinophilic, cytoplasm; at

TABLE I: EFFECT OF THE FEEDING OF RIBOFLAVIN ON THE PRODUCTION OF LIVER CHANGES BY *p*-DIMETHYLAMINOAZOBENZENE IN RATS ON A PURIFIED DIET FREE FROM RIBOFLAVIN

No riboflavin				30 mgm. riboflavin per week			
Autopsy number	Final weight, grams	Time on diet, days	Liver involvement	Autopsy number	Final weight, grams	Time on diet, days	Liver involvement
2661 *	97	40	1+	1854	210	64	o
2662 *	68	40	1+	1929	265	80	o
2683 *	56	40	1+	1940 †	231	88	o
2684 *	63	40	1+	1976	224	92	o
2717 *	70	41	2+	3042 *	154	99	o
2718 *	76	43	2+	2051 †	223	108	o
2665 *	64	43	2+	2054	365	111	o
2719 *	74	44	2+	3098 *	207	120	±
2720 *	76	45	2+	3101 *	286	120	±
2666 *	86	53	±	3102 *	142	120	1+
2722 *	86	53	2+	2165 †	318	142	o
2725 *	92	56	1+	2256	362	177	o
1853	78	64	±	2257	388	177	o
1855 †	70	64	1+	2259	429	178	o
1920	86	78	2+	2260	327	178	o
1977	65	92	2+	3620 *	344	258	1+
1978	69	92	1+	3621 *	462	258	1+
1943	66	99	1+	2628	415	316	±
2000	82	100	2+	2699	465	338	2+
2053	111	111	2+	2700 †	293	338	2+
2055	82	111	2+	2701 †	360	338	2+
3275 *	152	149	1+	4008 *	400	342	3+
2186	88	160	2+	4009 *	301	342	2+
2187	122	160	2+	2639 ‡	164	316	2+
2238	130	169	3+	2640 ‡	240	317	2+
2239	115	169	3+	2702 ‡	222	338	3+

* *p*-Dimethylaminoazobenzene was added to the diet when the rats were 6 weeks old.
† These animals received 50 mgm. of nicotinic acid 3 times a week.
‡ These animals were deprived of riboflavin after 160 days.

times they were coarsely vacuolated. Some of the liver cells contained 2 or more nuclei. The cells abutting on the periportal fields as well as on the central veins were, for the most part, smaller. The Kupffer cells were prominent. Bile duct proliferation was occasionally noted. Scattered within the cellular areas mentioned above, isolated islands of liver tissue were present which did not show a striking difference from the uninvolved liver tissue, except, perhaps, in the presence of a greater number of the larger cells with more basophilic cytoplasm.

The livers graded 3+ showed carcinoma.

In the absence of riboflavin, the feeding of *p*-dimethylaminoazobenzene produced considerable hepatic damage in all animals. These changes were noted in some (not listed in the table) as early as 25 days after feeding of the dye was begun. Supplementing with nicotinic acid was ineffective in checking the liver damage caused by administration of *p*-dimethylaminoazobenzene. Likewise, the feeding of gum acacia in amounts of 0.5 cc. 3 times a week failed to influence the occurrence of pathologic changes in the liver.

The administration of 10 mgm. of riboflavin 3 times a week protected all animals from liver damage for a period of at least 4 months. However, supplementary riboflavin continuously administered failed to prevent the liver damage when the animals were exposed to *p*-dimethylaminoazobenzene for more than 5 to 6 months. Thereafter, histologic examination revealed severe involvement of the liver in all animals.

The protective effect of riboflavin in our rats receiving ample amounts of casein and choline in the basal diet consisted, therefore, in retarding the occurrence of liver damage resulting from *p*-dimethylaminoazobenzene for a period of about 5 months. During this period all animals receiving no riboflavin developed liver damage varying in degree from degenerative changes to carcinoma. After the 5 months' period of apparently complete protection, pathologic changes of the liver took place which were comparable to those observed in the riboflavin-deficient animals.

To judge from the degrees of hepatic damage noted, the initial change is of a degenerative nature which subsequently takes on a proliferative tendency terminating in carcinoma. In the prevention of liver damage, riboflavin may play its protective role by impeding the degenerative changes and thus checking indirectly the proliferative changes. It may have a dual action, since in addition to impeding degeneration, it may inhibit proliferation. This latter possibility is unlikely, however, since riboflavin neither prevents the production of benzpyrene tumors in rats (unpublished experiments of the authors) nor stops the growth of transplants of sarcoma 180 in mice (unpublished observations, Wm. Antopol and S. Glaubach).

CONCLUSION

The administration of large amounts of riboflavin (10 mgm., 3 times per week) retards the occurrence of pathologic changes in the liver produced by *p*-dimethylaminoazobenzene in rats maintained on a synthetic diet free from riboflavin and nicotinic acid; nicotinic acid does not have this effect.

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Cancer Res 1942;2:694-696.