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Influence of Riboflavin Antagonists on Azo Dye Hepatoma Induction in the Rat[1] (39585)

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In 1936, Kinosita (1) observed that feeding a diet containing 4-dimethylaminoazobenzene (DAB) caused the induction of hepatomas in rats. Kensler and co-workers (2) showed that excess riboflavin (Fig. 1, A) provided such experimental rats with protection against the carcinogenic action of the dye. Griffin and Baumann (3) and Miller and co-workers (4, 5) found that a decrease in riboflavin content of the liver occurred when DAB or 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) was added to the diet.

An azo reductase in the rat liver has been described (6, 7). The administration of DAB causes a decrease in the activity of this enzyme to cleave the carcinogen (8, 9) while the administration of excess riboflavin has been reported to enhance the activity of this enzyme (6, 8). However, evidence that this azo reductase is a flavoprotein has not been convincing.

More recently, Lambooy (10) has shown that when a critical balance has been struck between the quantities of 3'-Me-DAB and riboflavin administered, most of the rats (87%) do not develop hepatomas during 18 weeks, while some (13%) do. It was further shown that if in place of riboflavin, one or another of two vitamin-like homologs (Fig. 1, B and C) of riboflavin, both of which were equivalent to riboflavin in terms of growth, efficiency of food utilization, and survival (11), but both of which cause reduction of some enzymic activity (12, 13), was used, approximately 90% of the experimental animals developed hepatomas or precancerous conditions. Thus, restriction of the amount of exogenous riboflavin enhances while increased amounts inhibit hepatoma formation when DAB is administered. Further, riboflavin analogs possessing less than complete vitamin-like replacement value for the vitamin, do not protect the rat when 3'-Me-DAB is administered.

There remains one more mechanism by which the role of riboflavin in the hepatoma induction process can be evaluated and that is to reduce the effectiveness of riboflavin through the simultaneous administration of potent antagonists of riboflavin. The most potent antagonists of riboflavin are 7,8-diethyl-flavin (Fig. 1, D) (14), a competitive antagonist of the vitamin, 7-chloro-8-methyl-flavin (Fig. 1, E) (15), a riboflavin analog possessing mixed vitamin-like and antagonist properties, and 7,8-diethyl-aminol-flavin (Fig. 1, F) (to be published in J. Med. Chem.), a riboflavin antagonist which, while still a flavin, possesses a side chain at position 10 other than D-ribityl. This report describes the effects that these antagonists have on the carcinogenic process induced by 3'-Me-DAB and how these findings provide additional support for the role of riboflavin in this process.

Materials and methods. Male rats of the Sprague-Dawley strain were used.[2] They were obtained from the supplier weighing 128-154 g; they were divided into five groups of 20 animals each and were housed two to a cage in quarters maintained under continuous temperature (24 ± 2°) and illumination (12 hr:12 hr) control. The rats were fed the following basic diet *ad libitum*. Vitamin B-complex free test diet[3] was used, to each kilogram of which were added (milligrams): pteroylglutamic acid, 0.6; biotin, 1.5; thiamine-HCl, 20; pyridoxine-HCl, 20; riboflavin, 2; nicotinamide, 50; potas-

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[2] CFE rats from Carworth Division, Charles River Breeding Laboratories, Wilmington, Mass. 01887.

[3] Sucrose 68%, casein 18%, corn oil 10%, salt mixture II 4%; obtained from Teklad Test Diets, Madison, Wis. 53711.

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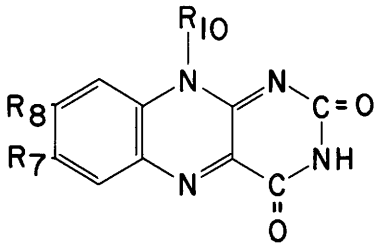

FIG. 1. Basic flavin structure.

Flavin	R_7	R_8	R_{10}	Trivial name
A	CH_3—	CH_3—	—CH_2—$(CHOH)_3$—CH_2OH	Riboflavin
B	C_2H_5—	CH_3—	—CH_2—$(CHOH)_3$—CH_2OH	7-Ethyl-8-methyl-flavin
C	CH_3—	C_2H_5—	—CH_2—$(CHOH)_3$—CH_2OH	7-Methyl-8-ethyl-flavin
D	C_2H_5—	C_2H_5—	—CH_2—$(CHOH)_3$—CH_2OH	7,8-Diethyl-flavin
E	Cl—	CH_3—	—CH_2—$(CHOH)_3$—CH_2OH	7-Chloro-8-methyl-flavin
F	C_2H_5—	C_2H_5—	—$(CH_2)_2$—$N(CH_2$—$CH_2OH)_2$	7,8-Diethyl-aminol-flavin

sium *p*-aminobenzoate, 50; calcium pantothenate, 60; inositol, 100; and (micrograms) cyanocobalamin, 40. To the diet was also added 1.5 g of choline chloride, and 20 g of cod liver oil containing 50 mg of menadione and 1 mmole (239 mg) of 3'-Me-DAB. Group A was fed the basic diet; Group D was fed the basic diet to which was added[4] 10.2 mg/kg of 7,8-diethyl-flavin (Fig. 1, D); Group E-I was fed the basic diet to which was added[4] 10.0 mg/kg of 7-chloro-8-methyl-flavin (Fig. 1, E); Group E-II was the same as Group E-I except that the analog was reduced to 5.0 mg/kg of diet; Group F was fed the basic diet to which was added[4] 11.0 mg/kg of 7,8-diethyl-aminol-flavin (Fig. 1, F). The groups were fed their respective diets for a period of 18 weeks after which time the animals were killed and their livers were examined for hepatomas or other gross changes.

Results and discussion. Feeding a diet containing 2 mg of riboflavin and 1 mmole of 3'-Me-DAB/kg of otherwise adequate composition to rats, protected the livers of these animals against hepatoma formation to the extent that 80% were found to be normal (Group A). These findings confirm our earlier studies (10).

When animals (Group D) were fed the above basic diet to which had been added the riboflavin antagonist flavin D, the beneficial effect of the riboflavin was suppressed as is evidenced by 80% of the animals developing hepatomas and 20% showing precancerous conditions. The antagonistic activity of the 7,8-diethyl-flavin is also illustrated by the significant reduction in the rate of growth of the animals receiving it.

When animals (Group E-I) were fed the above basic diet to which had been added flavin E, a flavin possessing both vitamin-like and antagonist activities, 30% of the animals died between the third and eighth week of the experiment. None of the animals that died possessed hepatomas at the time of death. As had been observed before (15), large quantities of this analog emphasized its antagonist properties. Only 25% of the livers of this group were found to be normal while 45% showed hepatomas (35%) or precancerous lesions (10%). When the immediately preceding experimental group was reconstituted but with the quantity of flavin E reduced to 5.0 mg/kg (Group E-II), all the animals survived, and furthermore, some of the vitamin-like properties of the analog were emphasized relative to the findings in Group E-I. For example, the gain in weight by this group was significantly greater than that of the control group (Group A). Only 11% of the animals in Group E-II developed hepatomas but the remaining 89% presented a gross appearance of livers not previously observed in our laboratories. The cirrhosis was severe but not unfamiliar, consisting of orange peel-like surface with deep segmentation and

[4] These quantities are equimolecular.

notching of the edges of all lobes. All lobes were "puffed" in appearance. The striking difference in appearance was the presence of very diffuse, very blanched, whitish area of the livers. These areas were not the same as those listed as small white lesions in Table I [previously identified as precancerous (16)], and they were distributed among all lobes. The larger lobes, the median lobe, and the right and left lateral lobes in some cases showed half of their surfaces to be of this blanched appearance. These areas were also observed in the livers of the two animals that had developed the tumors, but for rating purposes (Table I) the latter criterion was considered to be the more significant. These blanched, whitish areas were not observed in any of the four other groups.

When animals (Group F) were fed the above basic diet to which had been added flavin F, the results were totally unexpected. This flavin had been shown to be an antagonist of riboflavin in riboflavin-deficient rats. After the first 2 weeks of the experiment, the rate of growth of the animals of this group strongly suggested that this analog was supplementing riboflavin and the rate of growth continued to be greater than that of Group A throughout the 18-week experimental period. When the animals of Group F were killed and examined the livers of all were normal in appearance; there was no evidence of any abnormality.

Our extensive study of riboflavin analogs has firmly established that what is meant by "biological activity" of flavins is in need of considerable modification (see reference 14, pp. 358–362). In the case of the rat, we have shown that such biological activity might involve, to a considerable degree independently, growth, improved appearance, survival, reproduction, and inhibition of the beneficial influence of riboflavin. However, all of the above was based on observations made on flavins possessing the D-ribityl side chain at position 10. Our rationale (17) for the synthesis of flavins possessing the diethanolaminoethyl side chain at position 10, was to explore the possibility of developing a new class of antagonists of riboflavin. We had not anticipated that such an extreme departure from the normal side chain could be associated with vitamin-like activity. The particular details of the differences between two of the flavins used must be emphasized. Flavin D differs from the vitamin only in that it possesses ethyl groups in place of methyl groups at positions 7 and 8; it is clearly an antagonist of riboflavin in this system. Flavin F differs from the vitamin in that the ethyl groups have replaced the methyl groups at positions 7 and 8, but

TABLE I. INCIDENCE OF HEPATOMAS IN RATS FED RIBOFLAVIN, OR RIBOFLAVIN WITH VARIOUS RIBOFLAVIN ANALOGS, AND 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE.

Flavin	Starting wt (g)	Wt gained (g)[a]	Liver findings				
			Normal[b]	Tumors[c]	Small lesions[d]	Cirrhosis[e]	Died
A	141 ± 2	342 ± 11	16/20[f] (80)		4/20 (20)		
B	142 ± 1	289 ± 10[g]		16/20 (80)	3/20 (15)	1/20 (5)	
E-I	143 ± 2	337 ± 18	5/20 (25)	7/20 (35)	1/20 (5)	1/20 (5)	6/20 (30)
E-II[h]	141 ± 2	430 ± 17[i]		2/19 (11)		17/20[j] (89)	
F	142 ± 1	394 ± 12[k]	20/20 (100)				

[a] Average weight gained by the surviving animals during the 18-week period ± SE.

[b] Normal in terms of visual gross inspection.

[c] Tumors varied from one to many; from 4 mm to 3 cm in diameter; these livers were cirrhotic.

[d] Small lesions 1 to 3 mm in diameter, whitish in color, not yet grossly nodular, but clearly precancerous. These livers also showed cirrhosis.

[e] Cirrhosis, but no small lesions nor tumors.

[f] Number of animals showing change over the number of animals in the group. In parentheses is the percentage of the animals in the group showing change.

[g] The P value (Student's t test) for the difference between this weight and that of the riboflavin group = 0.0008.

[h] This group started 4 weeks after the other groups.

[i] The P value for the difference between this weight and that of the riboflavin group = 0.0001.

[j] In addition to the severe cirrhosis, see text for description of appearance.

[k] The P value for the difference between this weight and that of the riboflavin group = 0.0024. The difference between this weight and that of Group E-II was not considered significant (P = 0.100).

further, the side chain at position 10 is greatly different from the normal D-ribityl. This study provides very convincing evidence that flavin F possesses vitamin-like properties in this test system. However, there are alternative possibilities which can be mentioned but not supported at this time.

If the azo reductase is important in protection against the induction of hepatomas by DABs, one might speculate that the flavin F stimulates its formation or activity, either of which role suggests vitamin-like activity, although only in a very limited and local sense. Flavin F might be destroyed preferentially with the end result that the spared riboflavin is responsible for the apparent supplemental action of the analog. Still another possibility is that flavin F may simply protect the liver against the effects of 3′-Me-DAB by mechanisms unknown at present. All of the above mechanisms or possibilities are susceptible to study.

The influence of riboflavin on the induction of hepatomas in rats receiving DABs has now been demonstrated by all possible means available.

Summary. The addition of a small amount of riboflavin to an otherwise adequate diet, but a diet containing the hepatic carcinogen 3′-methyl-4-dimethylaminoazobenzene, strongly inhibits the carcinogenicity of the dye when fed to rats for 18 weeks. Most of the rats (80%) did not develop hepatomas. When the riboflavin antagonist 7,8-diethylflavin was also added to the above basic diet, all of the animals developed tumors (80%) or precancerous lesions (20%). When 7-chloro-8-methyl-flavin was added to the above basic diet, which of its two properties, the vitamin-like or the riboflavin antagonist properties, predominated depended on the quantity administered, but the flavin did not protect the livers from gross abnormalities. When the flavin 7,8-diethyl-aminol-flavin was added to the above basic diet, none of the animals developed hepatomas, suggesting that this flavin possesses strong vitamin-like properties or that it is capable of protecting the liver against the effects of the carcinogen.

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