Riboflavin Protection Against Azo Dye Hepatoma Induction in the Rat[1] (34757)

JOHN P. LAMBOOY

The Department of Biological Chemistry, the School of Medicine, the University of Maryland at Baltimore, 21201

In 1936 Kenosita demonstrated that feeding a diet containing 4-dimethylaminoazobenzene (DAB) caused the production of hepatomas in the rat. Five years later, Kensler *et al.* (1) showed that excess riboflavin provided such experimental rats with protection against the action of this dye and this observation appears to have been the first evidence suggesting that nutrition might play a role in carcinogenesis. The *in vitro* activity of a liver azo reductase enzyme is enhanced by the addition of excess riboflavin (2, 3) and the administration of DAB causes a decrease in the ability of this enzyme to cleave DAB (4, 5). The evidence that this enzyme is a flavoprotein has not been convincing.

Morris and Robertson (6) found that the growth rate of mammary carcinomas in C3H mice was decreased by severe riboflavin deficiency. Holly *et al.* (7) reported that the riboflavin analog 6,7-dichloro-9-(1′-D-sorbityl)-isoalloxazine, which has no antiriboflavin activity in rats, caused regression of established lymphosarcoma in mice.

More recently Kim *et al.* (8) showed that 7-ethyl-8-methyl-10-(D-ribityl)isoalloxazine (7-Et-flavin), a homolog of riboflavin, specifically inhibited the growth rate of Walker rat carcinoma 256. The latter compound is not a riboflavin antagonist in any test system tried; it is able to serve as the sole flavin for *Lactobacillus casei* and for the rat (9). While an isomer of the above 7-Et-flavin, namely 7-methyl-8-ethyl-10-(D-ribityl)isoalloxazine (8-Et-flavin) is nearly as potent a replacement of riboflavin for the rats as 7-Et-flavin, it exerted no inhibitory action on the growth rate of the Walker carcinoma.

At the cellular level, in the liver of the rat, a striking effect of the activities of these two homologs is observed. The 7-Et-flavin causes a very rapid and extreme depression (to 18% of normal in 120 days) in the succinic acid dehydrogenase activity (10) while 8-Et-flavin does not cause as great a drop (to 60% of normal in 120 days) in the activity of this enzyme. The flavin contained by the livers of the rats given 7-Et-flavin or 8-Et-flavin was, of course, the flavin administered, and in both cases the concentrations of the flavins were the same as that of animals receiving riboflavin (11).

Recently Bebawi and Lambooy (12) synthesized a series of new prime-ring, disubstituted DABS. These materials showed no inhibitory activity against *L. casei* but they were all found by Bebawi *et al.* (13) to be carcinogenic, and one, 3′-methyl-4′-ethyl-4-dimethylaminoazobenzene (3′-Me-4′-Et-DAB) was found to be an exceptionally potent carcinogen.

The availability of two flavins which are able to satisfy completely the rat requirement for riboflavin and yet cause reduced enzyme activity in the liver provided us with an opportunity to investigate the role of riboflavin in carcinogenesis in a new way. Previous work showing the protective action of riboflavin on hepatoma formation made use of the usual quantity of DAB, 2′-Me-DAB or 3′-Me-DAB (usually 0.06% of the diet) with diets containing either low or high concentrations of riboflavin. It was our opinion that the great differences in riboflavin content of the diets had the effect of testing the dye in two different classes of livers, in a biochemical sense. Characteristically, protection against DAB by this means was complete but

[1] This work was supported in part by Research Grant No. T-441 from the American Cancer Society.

equal protection against 2′-Me-DAB and 3′-Me-DAB by excess riboflavin has not been demonstrated. We found that by the use of a considerably reduced quantity of dye and the quantity of riboflavin (2 mg/kg of diet) usually used when carcinogenesis is desired, we could induce hepatomas in a small percentage of the experimental animals. Thus, if the homolog flavins, 7-Et-flavin and 8-Et-flavin were to show the results of a reduced flavin metabolism in the liver, and if this has anything to do with azo dye induced carcinogenesis, the same quantity of dye should produce an increased rate of hepatoma induction. This was found to be the case.

Once having found the proper combination of riboflavin and 3′-Me-DAB which would result in some, but only a low incidence of hepatomas, this quantity of dye was used with equivalent amounts of each of the three flavins. A similar study was undertaken using the same quantities of the flavins and an amount of 3′-Me-4′-Et-DAB, equivalent to the quantity of 3′-Me-DAB used above. In the latter study the small quantity of riboflavin used provided no protection against the carcinogen. This confirms our earlier results based on work done by different procedures, that 3′-Me-4′-Et-DAB is a more potent carcinogen than 3′-Me-DAB.

Materials and Methods. Male rats of the Sprague-Dawley strain were used.[2] They were obtained from the supplier weighing 160–175 g, divided into six groups of 16 animals each and housed 2 to a cage in quarters maintained under continuous temperature control (75 ± 1°). The rats were fed the following basic diet *ad libitum*. Vitamin B-complex text diet,[3] to each kg of which were added (g): cod liver oil, 20; choline chloride, 1.5; and (mg): pteroyglutamic acid, 0.6; biotin, 1.5; thiamine–HCl, 20; pyridoxine–HCl, 20; menadione, 50; nicotinamide, 50; K-*p*-aminobenzoic acid, 50; Ca pantothenate, 60; inositol, 100 and cyanocobalamin, 40 μg. To two lots of the above basic diet was added 2 mg of riboflavin; to one of these lots was added 1 mmole/kg of 3′-Me-DAB (Diet Rb, 3′) and to the other 1 mmole/kg of 3′-Me-4′-Et-DAB (Diet Rb, 3′,4′). To two lots of the above basic diet was added 4.4 mg[4] of 7-Et-flavin; to one of these lots was added 1 mmole/kg of 3′-Me-DAB (Diet 7-Et, 3′) and to the other 1 mmole/kg of 3′-Me-4′-Et-DAB (Diet 7-Et, 3′,4′). To two lots of the above basic diet was added 5.7 mg[4] of 8-Et-flavin; to one of these lots was added 1 mmole/kg of 3′-Me-DAB (Diet 8-Et, 3′) and to the other 1 mmole/kg of 3′-Me-4′-Et-DAB (Diet 8-Et, 3′,4′). The above six diets were fed to corresponding groups of rats for a period of 18 weeks at which time the animals were killed and their livers examined for hepatomas or other gross changes. The results are summarized in Tables I and II.

TABLE I. Incidence of Hepatomas in Rats Fed the Three Flavins and 3′-Methyl-4-dimethylaminoazobenzene.

Flavin	Wt gain (g)[a]	Tumors	Cirrhosis	Normal
Rb	176	2/13[b]		14/87
7-Et-flavin	115	12/75	2/13	2/13
8-Et-flavin	142	10/62	5/31	1/6

[a] Average weight gained by the animals during the the experimental period (starting wt 160 to 175 g).

[b] The number of animals over the percentage of the animals in the group.

TABLE II. Incidence of Hepatomas in Rats Fed the Three Flavins and 3′-Methyl-4′-ethyl-4-dimethylaminoazobenzene.

Flavin	Wt gain (g)[a]	Tumors	Cirrhosis	Normal
Rb	132	16/100[b]		
7-Et-flavin[c]	98	16/94	1/6	
8-Et-flavin	99	13/81	3/19	

[a] Average weight gained by the animals during the experimental period (starting wt 160 to 175 g).

[b] The number of animals over the percentage of the animals in the group.

[c] This group consisted of 17 animals.

[2] CFE rat from Carworth, New City, New York.

[3] General Biochemicals, Chagrin Falls, Ohio.

[4] These quantities are equivalent to 2 mg of riboflavin/kg of diet in terms of growth and utilization of food; Ref. (9).

Results and Discussion. The results shown in Tables I and II require a minimum of clarification and discussion. Both experiments emphasize the fallacy of trying to compare carcinogenicities of azo dyes by mixing the materials in the diet. If no dye had been added to the diets, all six groups of animals would have consumed the same amounts of diet and shown the same average weight gains. The animals in Group Rb, 3′,4′ did not eat as much diet as the animals in Group Rb, 3′, and as a result they did not gain as much weight nor did they ingest as much dye. In this particular instance the unequal ingestion of dye had no influence on the determination of the relative carcinogenicities of the two dyes. When these two dyes were administered by stomach tube each day (3′-Me-DAB in twice the amount as 3′-Me-4′-Et-DAB) no difference in food consumption or growth rate was observed (14).

Both groups of animals receiving 3′-Me-DAB and the two riboflavin homologs showed greatly increased incidence of hepatomas over the groups receiving the same dye but with riboflavin. This was true in spite of the reduced diet consumption and, therefore, the reduced dye ingestion by these two groups. Had the diet consumption been the same, it is not unreasonable to expect that the incidence of hepatomas might have been increased.

The same can be said concerning the diet consumption by the three groups receiving the 3′-Me-4′-Et-DAB. In this case it would be erroneous to conclude that the two riboflavin homologs provided more protection against the dye than riboflavin. If the diet consumption had been the same it is reasonable to expect that all the animals in the three groups would have shown hepatomas. These animals showing "cirrhosis" would have very likely developed tumors if the experimental period had been lengthened.

It is evident that the animals in both experiments which received riboflavin homologs were less able to combat the ill effects of the dyes, particularly those which were responsible for the reduced food consumption, than the groups which received the riboflavin. This must have been due to some abnormality in the metabolism of the riboflavin-requiring process but these effects need not necessarily be limited to the liver nor to succinic acid dehydrogenase, for example.

These studies have added considerable support for the role of riboflavin in the hepatogenesis process induced by the azo dyes. The fact that the two riboflavin homologs used produced quite different responses in the succinic acid dehydrogenase activities in the liver while the hepatoma incidence in each experiment was essentially the same tends to rule out this enzyme as one of primary responsibility in the process. The influences of these riboflavin homologs on other enzymes are under investigation.

The earlier finding that 3′-Me-4′-Et-DAB is a far more potent carcinogen than 3′-Me-DAB had been abundantly confirmed.

The author acknowledges with appreciation the technical assistance provided by Miss Kathleen Ann Lambooy.

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Received Jan. 16, 1970. P.S.E.B.M., 1970, Vol. 134.