

CORRELATION OF THE LEVEL OF HEPATIC RIBOFLAVIN
WITH THE APPEARANCE OF LIVER TUMORS IN RATS
FED AMINOAZO DYES*

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The effect of structure on the carcinogenicity of aminoazo dyes related to 4-dimethylaminoazobenzene (1-4) and the influence of various diets on the activity of 4-dimethylaminoazobenzene (5-7) have been the subjects of extensive study. As a result experimental conditions have been defined which make it possible to obtain a wide variation in the incidence of hepatic tumors by selecting the proper carcinogen and diet. Since no extrinsic factors, such as destruction of the dye in the diets (6), appeared to be responsible for the variations in the rate of tumor formation, a search for possible metabolic changes related to carcinogenesis was begun. Most of the initial experiments with this approach have been inconclusive. Thus, rats fed 4-dimethylaminoazobenzene in protective diets usually maintain levels of free aminoazo dyes in the blood and liver which are almost as high as those found in rats on non-protective diets (8). Since most aminoazo dyes, irrespective of their carcinogenicity, are dealkylated by the rat (1, 2, 9) the *in vivo* lability of the *N*-alkyl groups of the aminoazo dyes is not necessarily associated with the carcinogenic process. Furthermore, the over-all metabolism of 4-dimethylaminoazobenzene by rats fed various diets is similar since approximately the same levels of the same monophenyl amine metabolites are excreted in the urine irrespective of the tumor-promoting or inhibiting nature of the diet (10). However, rats fed a protective diet high in riboflavin do metabolize 4-dimethylaminoazobenzene differently from those fed diets promoting tumor induction in at least one respect, since the livers of rats on the high riboflavin diet contain less protein-bound aminoazo dye throughout the period of carcinogenesis than precancerous livers (11).

Kensler and his coworkers (12) and Griffin and Baumann (13) have observed a decrease in the level of hepatic riboflavin in the livers of rats fed certain carcinogenic aminoazo dyes. In the present paper the levels of riboflavin,

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biotin, and vitamin B₆ were determined in the livers of rats fed 4-dimethylaminoazobenzene in 10 different protective and non-protective diets for 6 and 19 weeks; similar determinations were also made on the livers of rats fed some of the same diets without the carcinogen. In addition similar analyses were performed on the livers of rats fed the dye in diets containing either urethane or increased levels of cystine or methionine. Urethane produces lung tumors in rats (14, 15) and mice (16) and a few liver tumors in rats (14), but has been reported to exert no effect on the carcinogenicity of 4-dimethylaminoazobenzene (17). Varying activities have been assigned to extra dietary cystine and methionine (18-21). Finally, the concentration of riboflavin was determined in the livers of rats fed 4-dimethylaminoazobenzene and 4 of its 5 possible C-monomethyl derivatives at several times during a 16 week period. All the data indicate that rats with a high level of hepatic riboflavin are much less likely to develop liver tumors than animals with a lower concentration, while the levels of hepatic biotin and vitamin B₆ are not correlated with the tumor incidence.

Methods

Care of the Animals.—Male Sprague-Dawley rats, 150 to 200 gm. in weight, were used exclusively. They were housed in screen bottom cages in groups of 6 to 8, and water was available *ad libitum*. The conditions used in each of the 4 series are described separately.

The object of series 1 and 2 was a comparison of the levels of certain vitamins in the livers of rats fed 4-dimethylaminoazobenzene in diets which either stimulate or inhibit the formation of hepatic tumors. The composition of the diets for series 1 is given in Table I; weighed amounts of these rations were fed so that the average food consumption for each group was 9 gm. per rat per day. Nine rats were fed each of the 10 diets with the addition of 0.06 per cent of 4-dimethylaminoazobenzene (dissolved in the oil of the diet, or, for the low fat diet, dissolved in acetone and evaporated on the diet) as well as diets 1, 3 to 5, and 7 to 10 in the absence of the dye. At least 3 animals from each group were sacrificed after 6 and 19 weeks, and their livers were analyzed for riboflavin, biotin, and vitamin B₆. In the case of tumor-bearing livers, the non-necrotic tumors and a sample of tumor-free (gross examination) liver were analyzed separately.

The control rats (group 11) in series 2 received diet 2, Table I, while groups 12, 13, and 14 received the same diet with either 0.2 per cent urethane, 0.3 per cent *dl*-methionine, or 0.5 per cent *l*-cystine, respectively, added at the expense of glucose. All the diets were fed *ad libitum*. Of the 18 rats in each group 3 were killed for analysis after 6 weeks; the livers of these animals and of 3 rats from the same stock (zero time controls) were analyzed for riboflavin and vitamin B₆. The remaining animals were examined by laparotomy at 17 weeks, continued on the same diets without the dye for an additional 8 weeks, and then killed for a final tumor count.

In series 3 and 4 the level of hepatic riboflavin was determined in rats receiving aminoazo dyes differing greatly in their carcinogenic potency. For both series the appropriate aminoazo dyes were added to diet 3, Table I; all diets were fed *ad libitum*. Series 3 consisted of groups of 18 rats fed either 0.054 per cent of 4-dimethylaminoazobenzene or 0.058 per cent of the 4'-methyl or 3'-methyl derivatives and groups of 7 which received either 0.058 per cent of 2-methyl- or 2'-methyl-4-dimethylaminoazobenzene or the basal diet (no dye added). Three rats from the same stock were killed at the beginning of the experiment, and 2 or 3 rats were

sacrificed after 1½, 3, 6, 12, and 16 weeks. Three groups of 20 rats fed either 0.054 per cent of 4-dimethylaminoazobenzene or equimolar amounts of the 4'-methyl and 3'-methyl derivatives comprised series 4. Two or 3 animals from each group were sacrificed at 1, 2, 3½, 6, 9, 13, and 22 weeks.

Analytical Methods.—Prior to analysis the rats were killed with ethyl ether, and the livers perfused *in situ* with 10 ml. of 2 per cent sodium citrate and 30 ml. of 0.85 per cent sodium chloride. Each liver was then homogenized in water in a Waring blender, and appropriate

TABLE I
Composition of Diets*†

Constituents	Diet No.									
	1	2	3	4	5	6	7	8	9	10
Casein (water-extracted), gm.	120	120		120	120	120	120	120		120
Casein (crude), gm.....			120							
Egg white (ethanol-extracted), gm.....									120	
Corn oil, gm.....	50	50	50	200		50	50		50	50
Hydrogenated coconut oil, gm.								50		
Glucose monohydrate, gm...	790	790	770	640	840	790	790	790	790	788
Salt mixture, gm.....	40	40	40	40	40	40	40	40	40	40
Detergent (penetrant 7), gm.										2.5
Rice-bran extract (vitab), gm.			20							
Riboflavin‡, gm.....	0.002	0.001	0.001	0.002	0.002	0.005	0.010	0.002	0.002	0.002

* Each diet except No. 3 also contained 2.5 mg. pyridoxine hydrochloride, 3.0 mg. thiamine hydrochloride, 7.0 mg. calcium pantothenate, and 300.0 mg. choline chloride per kilo.

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‡ The dietary constituents were analyzed for riboflavin and then sufficient crystalline vitamin was added to bring the total content to the level given.

aliquots were taken for each analysis. Riboflavin was determined by the method of Conner and Straub (22) after neutralization of the acid hydrolysate with sodium acetate and incubation with papain for 20 hours. The enzyme treatment increased the values by about 10 per cent above those obtained after acid hydrolysis alone. Vitamin B₆ and biotin were assayed through the growth of *Saccharomyces carlsbergensis* and *S. cerevisiae*, respectively, according to the general method of Atkin and his associates (23).¹ The results are expressed as micrograms per gram of fresh liver.

RESULTS

Effect of Dietary Variations.—In general the livers of rats fed the diets containing 4-dimethylaminoazobenzene contained only about two-thirds as much

¹ We are grateful to Mrs. H. N. Kingsley for the biotin and vitamin B₆ analyses.

riboflavin as the livers of rats on the same diets without the dye (Table II). For example, the livers of the control group (group 1) fed the dye averaged

TABLE II
*The Levels of Riboflavin, Biotin, and Vitamin B₆ in the Livers of Rats Fed Protective and Non-Protective Diets. Series 1**

(3 to 6 rats per group; analyses at 19 weeks)

No.	Diet	Basal diet			Basal diet + 0.06 per cent 4-dimethylaminoazobenzene			Tumor incidence (6)	
		Riboflavin	Biotin	Vitamin B ₆	Riboflavin	Biotin	Vitamin B ₆		
		$\mu\text{g./gm.}$	$\mu\text{g./gm.}$	$\mu\text{g./gm.}$	$\mu\text{g./gm.}$	$\mu\text{g./liver}$	$\mu\text{g./gm.}$	$\mu\text{g./gm.}$	<i>per cent</i>
1	Control (2 mg. riboflavin/kg.)	20.4 (19-21)	0.90 (0.8-1.0)	10.9 (10-11)	13.7 (11-15)	121	0.53 (0.4-0.6)	7.8 (7-8)	50-80
2	Low riboflavin (1 mg./kg.)				9.8 (9-13)	95	0.58 (0.5-0.6)	7.6 (7-8)	90-100
3	Rice-bran extract	14.7 (14-15)	0.93 (0.9-1.0)	11.5 (11-12)	9.4 (9-10)	86	0.66 (0.4-1.0)	6.4 (6-8)	90-100
4	High corn oil	19.2 (17-23)	1.0 (0.9-1.1)	9.3 (8-11)	10.7 (9-13)	115	0.56 (0.5-0.7)	6.7 (6-10)	90-100
5	Low fat	19.6 (15-23)	0.58 (0.4-0.7)	11.5 (11-12)	15.2 (13-17)	110	0.58 (0.5-0.6)	7.4 (7-8)	20
6	Medium riboflavin (5 mg./kg.)				17.9 (17-18)	181	0.51 (0.5-0.6)	6.5 (6-8)	0
7	High riboflavin (10 mg./kg.)	28.6 (25-31)	1.23 (1.1-1.5)	8.7 (8-9)	19.4 (15-26)	174	0.72 (0.4-1.3)	7.5 (6-11)	0
8	Hydrogenated coconut oil	23.5 (21-27)	1.17 (1.1-1.3)	10.9 (10-12)	15.9 (12-21)	126	0.68 (0.5-1.0)	8.5 (7-10)	0-8
9	Egg white	23.6 (21-27)	0.90 (0.8-1.0)	10.4 (9-11)	17.4 (16-21)	156	0.37 (0.2-0.5)	6.0 (5-7)	0-14
10	Detergent	24.4 (23-26)	0.62 (0.5-0.7)	10.3 (9-11)	15.4 (14-17)	160	0.25 (0.2-0.3)	5.7 (5-7)	0

* The ranges for the animals in each group are given in parentheses; they are expressed to the nearest whole number for riboflavin and vitamin B₆ and to the nearest tenth for biotin.

13.7 $\mu\text{g.}$ of riboflavin per gm. as compared to 20.4 $\mu\text{g.}$ per gm. for those on the same basal diet; similarly, the livers of the rats on the high riboflavin diet (group 7) averaged 19.4 and 28.6 $\mu\text{g.}$ per gm. for the dye-fed and basal groups,

respectively. Further, of the rats fed 4-dimethylaminoazobenzene those which received protective diets had higher levels of hepatic riboflavin than those given diets accelerating tumor induction. Since essentially the same results were obtained at both 6 and 19 weeks, only the data for the later analyses are tabulated. The livers from rats on the control diet (group 1) averaged 13.7 μg . of riboflavin per gm. at 19 weeks; 50 to 80 per cent of the rats fed this diet usually develop liver tumors after 4 months on the dye and 2 months on the basal diet. The livers of rats fed diets which had either a low level of riboflavin, rice-bran concentrate as the source of vitamins, or 20 per cent of corn oil (groups 2 to 4) had 9.8, 9.4, and 10.7 μg . of riboflavin per gm. respectively; the tumor incidence on each of these diets usually approaches 100 per cent by the end of 4 months of dye-feeding. The level of hepatic riboflavin for those fed diets 6 to 10 averaged 15.2 to 19.4 μg . per gm. These diets contained either 5 or 10 mg. of riboflavin per kilo, hydrogenated coconut oil instead of corn oil, egg white in place of casein, or a synthetic detergent; each of these dietary alterations has, in the past, either prevented tumor development completely or allowed no more than 14 per cent of the rats to develop liver neoplasms by the end of 6 months. The livers of rats fed the low fat diet (group 5) contained 15.2 μg . of riboflavin per gm.; usually no more than 20 per cent of the animals on this diet have developed tumors. Essentially the same results were obtained when the total riboflavin per liver was calculated. The average total riboflavin content of the livers from rats fed each of the accelerating diets was lower than that of the control group (121 μg .), and all the groups fed protective diets other than the low fat diet had total hepatic riboflavin stores in excess of 121 μg .

As in the case of riboflavin the levels of biotin and vitamin B₆ were about two-thirds as high in the livers of rats fed 4-dimethylaminoazobenzene as in the livers of those fed the basal diets. However, in contrast to the above results, the levels of biotin and vitamin B₆ were not influenced appreciably by these dietary variations. At 19 weeks the levels of vitamin B₆ averaged from 5.7 to 8.5 μg . per gm. of liver for the 10 groups fed 4-dimethylaminoazobenzene. The levels of biotin averaged 0.51 to 0.72 μg . per gm. of liver for each of the first 8 groups, but dropped to 0.37 and 0.25 μg . per gm., respectively, when the egg white and detergent diets were fed.

Analysis of the tumors obtained from rats fed the control or accelerating diets confirmed earlier reports on their low vitamin content (24). Thus, the tumors contained an average of 4.4 μg . of riboflavin per gm. as compared to 9.8 μg . per gm. of the surrounding liver. The vitamin B₆ content averaged 2.5 μg . and 6.8 μg . per gm. for tumor and surrounding liver, respectively, while similar analyses for biotin were 0.12 and 0.52 μg . per gm., respectively.

The results of the vitamin analyses on series 2 are in good agreement with those from series 1. Thus, the rats receiving urethane developed tumors faster than any other group, and their livers had the lowest level of riboflavin,

7.9 $\mu\text{g.}$ per gm., at 6 weeks (Table III). The livers of the control group (1 mg. of riboflavin per kilo of diet) averaged 9.3 $\mu\text{g.}$ per gm., and the tumor incidence was 75 per cent at 6 months. Fifty per cent of the rats receiving 0.3 per cent *dl*-methionine developed tumors by 6 months while only 13 per cent of those supplemented with 0.5 per cent of *l*-cystine had neoplasms at this time; the livers from these groups averaged 10.7 and 10.9 $\mu\text{g.}$ of riboflavin per gm. at 6 weeks. The total hepatic riboflavin was 121, 84, and 69 $\mu\text{g.}$ per liver for the

TABLE III
The Levels of Riboflavin and Vitamin B₆ in the Livers of Rats Fed Protective and Non-Protective Diets. Series 2*
(3 rats per group; analyses at 6 weeks)

No.	Diet†	Riboflavin		Vitamin B ₆ $\mu\text{g./gm.}$	Tumor incidence	
		$\mu\text{g./gm.}$	$\mu\text{g./liver}$		4 mos. per cent	6 mos. per cent
Zero time		25.8 (24-26)	345	11.8 (11-13)		
11	Control	9.3 (9-10)	69	6.8 (6-7)	28	75
12	0.2 per cent urethane	7.9 (7-8)	59	6.1 (5-7)	47	80
13	0.3 per cent <i>dl</i> -methionine	10.7 (10-12)	84	8.4 (8-9)	33	50
14	0.5 per cent <i>l</i> -cystine	10.9 (10-12)	121	6.9 (6-7)	0	13

* See footnote to Table I.

† All diets contained 1 mg. of riboflavin per kg.

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ystine-supplemented, methionine-supplemented, and control rats, respectively. As in series 1 the level of vitamin B₆ could not be correlated with the tumor incidence. Our data are in substantial agreement with earlier reports which showed that cystine inhibited tumor induction to a significant extent (18-20), while methionine had little or no effect (21). The stimulation of the rate of tumor formation by urethane observed in this experiment is small and may not be significant.

Effect of Activity of Carcinogen.—When 4-dimethylaminoazobenzene was fed the level of hepatic riboflavin dropped rapidly from 22 $\mu\text{g.}$ per gm. at the beginning of the experiment to 15.6 $\mu\text{g.}$ at 1½ weeks and 10.4 $\mu\text{g.}$ at 3 weeks (Fig. 1). The riboflavin content continued to drop more slowly for the rest of the

experiment and reached 6.6 μg . per gm. at 16 weeks. A similar pattern was followed when each of the other aminoazo dyes was fed, but the extent of the decrease varied with the carcinogenicity of the compound. With 3'-methyl-4-dimethylaminoazobenzene, which is about twice as active as 4-dimethylaminoazobenzene, the liver riboflavin fell to 8.0 μg . in 3 weeks; at this time the livers of rats fed 2'-methyl-4-dimethylaminoazobenzene (one-third to one-half as active as 4-dimethylaminoazobenzene) contained 11.3 μg . per gm. Livers from rats fed 2-methyl-4-dimethylaminoazobenzene, which is inactive under our condi-

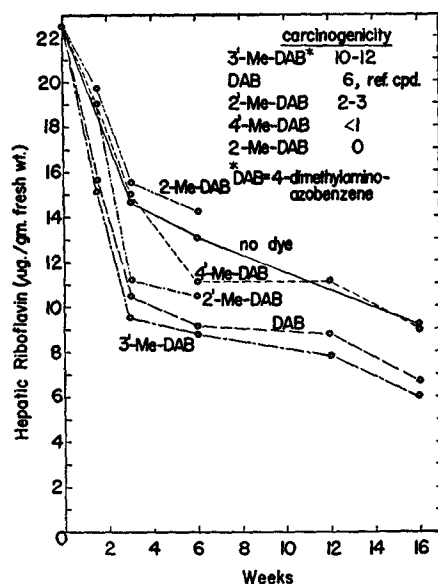


FIG. 1. The levels of riboflavin in the livers of rats fed 4-dimethylaminoazobenzene and its C-methyl derivatives.

tions, or 4'-methyl-4-dimethylaminoazobenzene, a very weak carcinogen, had approximately the same level of riboflavin as livers from animals on the basal diet. The progressive drop in the hepatic riboflavin of rats fed the basal diet is apparently a reflection of its minimal riboflavin content. Comparable results were also obtained in series 4. The riboflavin levels of the livers of rats fed 3'-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, and 4'-methyl-4-dimethylaminoazobenzene were 12.7, 13.5, and 16.0 μg . per gm. at 2 weeks and 8.2, 8.9, and 12.9, respectively, at 6 weeks.

DISCUSSION

The carcinogenic activity of the aminoazo dyes is related, at least in part, to the extent to which the level of hepatic riboflavin decreases when they are

fed. Thus, when various aminoazo dyes are fed in the same diet, the most rapid and greatest depression of riboflavin occurs in the livers of rats fed the most carcinogenic dyes. Similarly, the livers of rats fed 4-dimethylaminoazobenzene have lower levels of riboflavin when a non-protective diet is fed than when a protective diet is given; the levels of biotin and vitamin B₆, however, do not vary with the protective nature of the diet. The means by which the aminoazo dyes alter the riboflavin content of the liver is unknown, but alterations in the amount of intestinal synthesis, in the efficiency of absorption, or in the *in vivo* lability of riboflavin could give these results. There is little literature pertinent to the latter two possibilities, but some experiments suggest that the protective and accelerating diets could alter the amount of intestinal synthesis of riboflavin. Thus, Czaczkes and Guggenheim (25) found that rats fed a diet low in fat required less riboflavin daily to maintain their liver stores than animals receiving 10 per cent olive oil and those on a high fat diet required more riboflavin daily. From bacterial counts and riboflavin analyses on the feces they concluded that these effects were due to a greater synthesis of the vitamin by the intestinal flora in the presence of a low fat diet and a lesser synthesis with the high fat diet.

Since the level of hepatic riboflavin appears to be important in determining the resistance of the liver to 4-dimethylaminoazobenzene, the distribution of this vitamin in the liver cell has recently been investigated (26). The large granules (mitochondria) and the supernatant fluid from the homogenates of livers of rats fed the dye contained only 50 to 60 per cent as much riboflavin as the same liver fractions of rats on the basal diet, while the riboflavin content of the nuclei and small granules (microsomes) was not altered by the dye-feeding. Analyses of these fractions for total protein and nucleic acids also indicated extensive damage of the large granules by the dye.

The question still remains whether riboflavin interferes with a part of the carcinogenic process *per se* or whether it acts prior to this process. It is possible that riboflavin-containing enzymes are responsible for the destruction of the dye in the liver; thus livers with access to large amounts of the vitamin might destroy much of the dye before it has an opportunity to act as a carcinogen. For instance, Kensler (27) has found that slices of livers containing high levels of riboflavin destroy 4-dimethylaminoazobenzene faster than livers containing less of the vitamin. Preliminary data have also indicated that the protein-bound dyes are metabolized more rapidly by rats receiving high levels of dietary riboflavin (11). On the other hand, if riboflavin and 4-dimethylaminoazobenzene or their derivatives compete for a protein involved in the carcinogenic process, increased amounts of riboflavin would protect the protein and thus slow down the process of carcinogenesis. Unfortunately no data are available to support or disprove the latter idea.

SUMMARY

The livers of rats fed 10 diets previously found to inhibit or accelerate the induction of tumors by 4-dimethylaminoazobenzene were analyzed for riboflavin, biotin, and vitamin B₆ after 6 and 19 weeks on the diets. Those fed diets accelerating tumor induction had average hepatic riboflavin levels of 9 to 11 μg . per gm. The livers of the control and protected rats averaged 14 and 15 to 19 μg . per gm., respectively. The hepatic levels of vitamin B₆ and biotin averaged 7.0 and 0.54 μg . per gm., respectively, and were independent of the protective nature of the diet. The livers of rats fed the same diets in the absence of 4-dimethylaminoazobenzene contained about 50 per cent more of each of these vitamins than those from dye-fed rats.

In a second series the tumor incidence and the hepatic riboflavin and vitamin B₆ were determined simultaneously. In this experiment urethane decreased the level of hepatic riboflavin and increased the rate of induction of tumors slightly. Methionine inhibited tumor induction to a small extent while cystine had a more pronounced retarding effect; the level of hepatic riboflavin for both groups was higher than in the controls.

The livers of rats fed the basal diet, 0.054 per cent of 4-dimethylaminoazobenzene, or equimolar amounts of its 2-, 2'-, 3'-, or 4'-methyl derivatives were analyzed for riboflavin at various times over a 16 week period. In each case the level of hepatic riboflavin decreased throughout the experimental period, but the rate and extent of the riboflavin loss were greatest with the more carcinogenic compounds.

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