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Effect of dietary riboflavin on azo dye reductase in liver and in bacteria of cecal contents of rats

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THE EFFECT of riboflavin in modifying the carcinogenicity of certain azo dyes such as 4-dimethylamino azobenzene is now a classic phenomenon.[1] It received a sound biochemical explanation when Muell er and Miller[2] discovered that azo dye reductase in liver required FMN or riboflavin as a cofactor. Thus nutritional and exogenous factors which affected the level of azo dye reductase controlled at the same time the carcinogenic potency of the azo dyes.[1, 3]

A number of recent contributions have described the capability of bacterial flora in the gut of effecting the reduction of a number of azo dyes belonging mainly to the class of food colorants.[4–9] Additionally, we have demonstrated that carcinogens belonging to the class of aromatic amines were metabolized differently in germ-free and in conventional rats, and that the cecal bacterial flora was instrumental in modifying to an appreciable extent the course of metabolism of such agents.[10]

In the light of these developments, we have examined the possibility that the bacterial flora in the lower intestinal tract of rats might also participate in the metabolism of the carcinogenic azo dye, 4-dimethylaminoazobenzene. We found that the azo dye reductase specific activity in cecal contents was considerably higher than in liver, and that it was affected by the level of dietary riboflavin, although less so than the enzyme in liver.

MATERIALS AND METHODS

Treatment of animals. Young adult male Fischer rats were maintained for 6 weeks on the low casein (12%), low riboflavin (2 ppm) diet of Miller and Miller,[1] supplied by General Biochemicals, Inc., Chagrin Falls, Ohio 44022. Other groups of rats were maintained on the same diet to which additional riboflavin was added to give levels of 20 and 200 ppm respectively.

Assay procedures. During the fourth to sixth weeks of feeding, the rats were killed under light ether anesthesia. The livers were removed and stored in ice-cold containers. The cecum was isolated and the cecal contents removed and weighed.

Liver. The liver was minced with scissors. A 2-g aliquot was diluted with 4 vol. of 0·15 M phosphate buffer, pH 7·4, containing 1 μmole/ml each of $MgCl_2$ and NADPH. The mince was homogenized in a Potter–Elvehjem glass-Teflon apparatus and the homogenate centrifuged at 10,000 *g* for 20 min. Duplicate 1·0-ml aliquots of the supernatant fractions were taken, and either 0·1 ml buffer or 0·1 ml of a fine suspension of 1 μmole riboflavin was added. The tubes were flushed with oxygen-free nitrogen and substrate and 150 μg of 4-dimethylaminoazobenzene (a solution of 3 mg/ml in ethanol), was added. The stoppered tubes were incubated on a shaker at 37° for 30 min. The reaction was stopped by addition of 4 ml of 0·25 N NaOH, followed by five extractions of 5 ml of ether. The pooled ether extracts were taken to dryness under nitrogen. The residue was dissolved in 20·0 ml of acidified ethanol (995 ml ethanol and 5 ml 6 N HCl). The optical density at 520 nm was read and the concentration of the azo dye remaining determined, utilizing a standard curve which was linear from 0 to 6 μg dye per ml. There were samples without substrate and zero time samples as additional controls.

Cecal contents. The weighed cecal contents were diluted with 10 vol. of phosphate buffer, pH 7·4, containing 1 μmole/ml $MgCl_2$, and mixed gently by hand in a loose-fitting Potter–Elvehjem type instrument. To duplicate 1·0-ml samples, 0·1 ml of buffer or 0·1 ml of a fine suspension containing 1 μmole of riboflavin was added. The substrate, 4-dimethylaminoazobenzene (120 μg), was added and stoppered tubes were incubated for 1 hr at 37°. The reaction was stopped by addition of 2·0 ml of 0·5 N NaOH. Each tube was extracted five times with 5 ml ether and the azo dye in the ether extract evaluated after processing, as described for liver.

Protein samples of liver or cecal contents were assayed by the Fiszer modification[11] of the Lowry *et al.* method.[12]

RESULTS

The azo dye reductase activity per mg protein was considerably lower in liver than in cecal contents (Table 1). The activity of enzyme in the liver of rats fed the riboflavin-deficient Miller diet was increased appreciably when riboflavin was added *in vitro*. Likewise, the intestinal bacterial azo reductase from these rats was increased, but not as much as in the case of liver.

TABLE 1. AZOREDUCTASE ACTIVITY IN LIVER AND CECAL CONTENTS OF RATS FED VARIOUS LEVELS OF RIBOFLAVIN

No. of rats	Diet*	1 μmole/ml riboflavin added *in vitro*	DAB reductase specific activity† (nmoles/mg protein/30 min)	
			Liver	Cecal contents
8	M	No	3·3 ± 0·4	32·7 ± 3·9
		Yes	19·6 ± 3·1	49·9 ± 2·3
4	M^+	No	26·0 ± 4·9	38·4 ± 3·8
		Yes	32·8 ± 0·1	
4	M^{2+}	No	23·9 ± 1·1	50·5 ± 2·3
		Yes	28·7 ± 1·7	50·6 ± 2·3

* M: Miller diet,[1] 2 ppm riboflavin; M^+: Miller diet with 20 ppm riboflavin added; M^{2+}: Miller diet with 200 ppm riboflavin added.

† Values shown with standard errors of the mean and represent results of duplicate determinations on separate rats. DAB is 4-dimethylaminoazobenzene (*Chemical Abstracts* name: *N,N*-dimethyl-*p*-phenylazoaniline).

When the assays were performed on liver or cecal contents of rats on an intermediary level of riboflavin (20 ppm), they showed higher activity in the liver system but relatively little difference in the bacterial system. Supplementation *in vitro* with riboflavin raised the activity in liver somewhat. Enzyme activity in cecal contents of rats fed a 100-fold multiple of the minimal level of riboflavin, namely 200 ppm, showed even higher azo dye reductase activity which was not increased by the addition *in vitro* of riboflavin. With liver, similar limiting levels were found with 200 as with 20 ppm dietary riboflavin.

DISCUSSION

Our studies demonstrate that bacterial flora in cecal contents contributes measurably to the reduction of the azo bond of the carcinogen 4-dimethylaminoazobenzene. The data also reveal that not only the liver but also the bacterial flora exhibits lower enzyme activity in animals fed low riboflavin diets. Of interest in this connection is the fact that a 10-fold increase in the dietary riboflavin level (for the time period studied) does not appear to satisfy the vitamin requirements with regard to this particular enzyme system in cecum, since only a 100-fold increase gave maximal values of reductase.

It would appear that the participation of enzymes provided by the intestinal bacterial flora needs to be considered in studies of the overall fate of exogenous materials in mammalian systems, especially in cases where the compound is either fed orally or secreted into the intestinal tract via the bile. In addition to the example furnished by the present work, the report of Childs *et al.*[8] is relevant. 1-Phenyl-azo-2-naphthol was hepatocarcinogenic in mice when injected subcutaneously but not when fed orally, and the ability of the cecal contents of these mice to split the azo bond was demonstrated. Along the same lines, the toxicity and related effects of the food colorant, Brown FK, were highly dependent on enzymes of gut microflora.[13] The fat-soluble Prontosil is split by liver and by gut flora, whereas the more water-soluble Neoprontosil is reduced mostly by gut flora in rats.[14]

Thus, it is concluded that azo dye reductase which splits the carcinogen 4-dimethylaminoazobenzene is present in relatively large amounts in the bacterial flora of cecal contents of rats. The enzyme in cecal contents is depressed somewhat in rats fed low riboflavin, low protein diets, as is the enzyme

system from liver. Increasing the level of riboflavin to 200 ppm but not 20 ppm gives rise to maximal values of enzyme activity in cecal contents. Liver enzyme reached similar levels with 20 and 200 ppm supplementary dietary riboflavin.

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Comparative metabolism of selected *N*-methylcarbamates by human and rat liver fractions

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THE METABOLISM of *N*-methylcarbamates has been studied in plants,[1–3] vertebrate animals (*in vivo* and *in vitro*),[4–7] and insects.[8, 9] However, there is very little in the literature concerning their metabolism in the intact human or in isolated fractions of human liver. Matsumura and Ward[10] studied carbaryl metabolism by fractions from human livers obtained at autopsy. However, no attempt was made by these authors to identify the ether-extractable metabolites. Knaak *et al.*[6] investigated the metabolism *in vivo* of carbaryl by humans exposed to carbaryl dust by studying the urinary excretory products. They identified only the glucuronide and sulfate conjugates of 1-naphthol. More human studies are needed with many different types of compounds to increase our knowledge of the various metabolic pathways available (or not available) to the human and of possible differences between the human and other animals. The metabolism *in vitro* of the carbamates employed in this study by human liver fractions has not been investigated. The study, reported here, indicates there is a significant difference