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Reduction of Azo Dyes by Intestinal Anaerobes

KING-THOM CHUNG,†* GEORGE E. FULK, AND MARY EGAN

Chemical Carcinogenesis Program, National Cancer Institute Frederick Cancer Research Center, Frederick, Maryland 21701

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Reduction of seven azo dyes (amaranth, Ponceau SX, Allura Red, Sunset Yellow, tartrazine, Orange II, and methyl orange) was carried out by cell suspensions of predominant intestinal anaerobes. It was optimal at pH 7.4 in 0.4 M phosphate buffer and inhibited by glucose. Flavin mononucleotide caused a marked enhancement of azo reduction by *Bacteroides thetaiotaomicron*. Other electron carriers, e.g., methyl viologen, benzyl viologen, phenosafranin, neutral red, crystal violet, flavin adenine dinucleotide, menadione, and Janus Green B can replace flavin mononucleotide. These data suggest that an extracellular shuttle is required for azo reduction.

Azo dyes are widely used as colorants in foods such as soft drinks, candy, hot dogs, ice cream, and cereals and in drugs, cosmetics, etc. The extent of such use is related to the degree of industrialization of the society. For example, approximately 1.5×10^6 pounds (0.68×10^6 kg) of the dye amaranth were used each year in over 60 countries (9). It has been calculated that the annual American per capita consumption of these compounds is close to 5 pounds, which is twice the intake of 20 years ago (22).

Since intestinal cancer is more common in highly industrialized societies (2, 3, 23), a possible connection between these tumors and the use of azo dyes has been investigated (22). Some dyes have been reported to be toxic for animals; for example, amaranth has been shown to be toxic to the rat fetus (7, 8, 10) and carcinogenic in rats (1). Azo dyes are degraded by intestinal microorganisms in vivo (13, 14, 16), and it is possible that the toxic and/or carcinogenic effects of these dyes in the gut may be due to their degradation products. For example, 1-amino-2-naphthol, produced by the reduction of Orange II, has been reported to induce bladder tumors after bladder implantation (4, 5). Other dyes, such as Ponceau SX, Allura Red, Sunset Yellow, tartrazine, and methyl orange, are included in this study since they are similar in chemical structure and are widely used.

A few studies have been conducted to determine which bacterial species may be responsible for reducing and degrading the azo compounds. However, these experiments have been conducted with facultatively anaerobic enteric bacteria, which constitute only a small portion of the total intestinal microflora (15, 17, 19, 20).

This study was undertaken to determine the optimal conditions of azo reduction by intestinal anaerobes, since it may be important in generating carcinogenic/toxic compounds.

MATERIALS AND METHODS

Organisms. The organisms studied (Table 1) were obtained from the Anaerobe Laboratory, Virginia Polytechnic Institute (VPI), Blacksburg, except *Fusobacterium* sp. 2, which was obtained from Wadsworth Hospital Center, University of California, Los Angeles (6). The anaerobic techniques were those described in reference 12.

A round-bottom flask containing 500 ml of brain heart infusion medium (12) was inoculated with 1 ml of a pure culture grown in a chopped-meat broth and incubated at 37°C for 17 to 19 h. After incubation, the cells were centrifuged at 20,000 × *g* for 20 min and anaerobically washed once with 0.4 M potassium phosphate buffer (pH 7.4). The bacterial pellets were suspended in 25 to 40 ml of the same phosphate buffer and immediately used for azo reduction.

Materials. Ponceau SX, Allura Red, sodium-naphthionate, *m*-xylidine-6-sulfonic acid, and cresidine-sulfonic acid were kindly provided by Adrian B. Letherman of the Food and Drug Administration in Washington, D.C. Other compounds used were amaranth and sulfanilic acid (Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, N.J.), Sunset Yellow and methyl orange (Matheson, Coleman and Bell, Norwood, Ohio), tartrazine, 2,4,5-trimethylaniline, and *N,N*-dimethyl-*p*-phenylenediamine (ICN Pharmaceuticals, Inc., Life Sciences Group, New York, N.Y.), and Orange II, 2,4-dimethylaniline, HCl, 1-amino-2-naphthol HCl, and 2-amino-1-naphthalene sulfonic acid (Eastman Organic Chemicals, Eastman Kodak Co., Rochester, N.Y.).

The electron carriers used were flavin mononucleotide, methyl viologen, flavin adenine dinucleotide, and menadione (Sigma Chemical Co., St. Louis, Mo.), benzyl viologen (ICN), crystal violet and Janus Green B

† Present address: Food Sciences Institute, Purdue University, West Lafayette, IN 47907.

TABLE 1. *Reduction of azo dyes by intestinal anaerobes*

Organism	% Reduction (within 150 min)						
	Tartrazine	Ponceau SX	Sunset Yellow	Methyl orange	Orange II	Amaranth	Allura Red 40
Coprococcus catus (C20-4)	0	36	30	28	33	27	ND[a]
Acidaminococcus fermentans (C20-9)	4	6	9	66	72	5	0
Fusobacterium prausnitzii (C20-10)	2	0	4	80	74	30	33
Bacteroides thetaitaomicron (C20-14)	67	62	60	100	—[b]	53	18
Bifidobacterium infantis (C20-21)	0	0	0	31	59	30	13
Eubacterium biforme (C20-26)	4	29	22	79	81	19	11
Peptostreptococcus productus II (C20 28)	0	0	8	13	—	10	0
Citrobacter sp. (C20-36)	16	52	56	97	94	66	28
Peptostreptococcus productus I (C20-44)	6	13	23	62	64	42	0
Fusobacterium sp. 2 (Finegold CA-8/c-18)	100	100	100	100	100	100	100

[a] ND, Not determined.

[b] —, Reduction observed; quantitation was difficult due to the significant absorption of color by cells.

(Allied Chemical, Morristown, N.J.), neutral red (Matheson, Coleman and Bell), and phenosafranin (J. T. Baker Chemical Co., Phillipsburg, N.J.).

Azo reduction. One milliliter of dye (2 μmol/ml), 2 ml of cell suspension, and 2 ml of 0.4 M phosphate buffer (pH 7.4) with or without glucose and electron carriers were placed in roll tube (18 by 142 mm). The various electron carriers were prepared anaerobically at 0.025 mmol/ml. Each reagent was added anaerobically under nitrogen, using a VPI apparatus (12), and the reaction tubes were closed with rubber stoppers. Incubation was generally carried out at 37°C for 1 h, but the results given in Table 1 were obtained with a 2.5-h incubation. A zero-time control was also run for each dye.

The azo reductase is expressed as micromoles of azo dye disappearing per hour per 100 mg (dry weight) of cells.

Measurement of azo dyes. At the end of the incubation period, a 2-ml sample of the reaction mixture was added to 2 ml of 6% trichloroacetic acid and centrifuged at 29,000 × *g* for 20 min, and the clear supernatants were used for spectrophotometric measurements. The final dye concentration was within the reading range of the spectrophotometer (optical density, about 1.0). The absorption maximum of each dye was: amaranth, 520 nm; Sunset Yellow, 480 nm; Allura Red, 500 nm; tartrazine, 430 nm; Orange II, 480 nm; and methyl orange, 510 nm.

A standard curve for each dye was prepared by dissolving the dye in phosphate buffer and adding an equal volume of 6% trichloroacetic acid before spectrophotometric measurement. The blank was a solution consisting of equal volumes of the above 0.4 M phosphate buffer and 6% trichloroacetic acid.

Thin-layer chromatographic analysis. At the end of the incubation period, the remaining volumes of the reaction mixtures were centrifuged in an anaerobic hood, and the resulting supernatants were filtered through bacterial filters. The culture filtrates were kept under sterile anaerobic conditions until needed for thin-layer chromatographic analyses (without trichloroacetic acid added).

Thin-layer chromatographic analyses were made using Silica Gel G 250 (20 by 20 cm; Analtech, Inc., Newark, Del.) plates developed in four solvent systems (by volume): (i) methanol-chloroform (7:3), (ii) methanol-chloroform (7:6), (iii) methanol-chloroform-ammonium hydroxide (6:3:1), and (iv) methanol-chloroform-acetic acid (6:3:1), and visualized by Ehrlich reagent (12).

RESULTS AND DISCUSSION

The reduction of these dyes by different anaerobes is summarized in Table 1. The results show that all of the anaerobes tested reduce more than one azo dye. Some species (i.e., *Fusobacterium* sp. 2, *Bacteroides thetaiotaomicron,* and *Citrobacter* sp.) reduce most of the dyes listed, whereas others reduce only a few. The results indicate that the reduction of azo compounds can be accomplished by the major anaerobes rather than by a few facultatives in the gastrointestinal tract.

Metabolites, including sulfanilic acid, *N,N*-dimethyl-*p*-phenylenediamine, 1-amino-2-naphthol, *m*-xylidine-6-sulfonic acid, cresidine-sulfonic acid, sodium-naphthionate, and R-amino salt, are detectable by thin-layer chromatography, using authentic standard compounds for comparison. They were verified by their R_f value, color, and/or ultraviolet absorption on the plates. At least one reduction product for

each dye was visualized. Some suspected products, i.e., 2-amino-1-naphthol-4-aminopyrazolone from tartrazine, are not commercially available, and thus their identities were not verified. At least one other metabolite (either sodium sulphanilate or sodium naphthionate) from each dye was observed. The results show that there is cleavage of the azo linkage of these compounds.

Since anaerobes can reduce azo dyes, we determined the optimum conditions under which those reactions occurred. *B. thetaiotaomicron* (C20-14) was chosen for this study, since it is one of the predominant anaerobes in the gastrointestinal tract and is capable of reducing all the dyes listed in Table 1. Tartrazine was used as the substrate, since it is a common azo dye, and its reduction by *Proteus vulgaris* has been studied previously (17).

Since aeration of the reaction mixture caused a significant decrease in tartrazine reduction, all reaction mixtures were incubated anaerobically (under N_2) to facilitate the bacterial reduction.

Some dyes such as Orange II would not remain in solution during anaerobic incubation. Lowering the buffer concentration seemed to alleviate this problem. Different strengths of phosphate buffer were tested for optimum conditions, and maximum tartrazine reduction by *B. thetaiotaomicron* occurred in 0.4 M phosphate buffer. The pH is optimal at 7.4 to 8.5. This buffer concentration at pH 7.4 was used to generate the results given in Table 1 and was used in all subsequent experiments.

Roxon et al. reported that glucose stimulated the reduction of tartrazine by *P. vulgaris* (15). The effect of glucose on tartrazine reduction by *B. thetaiotaomicron* was inhibitory at concentrations as low as 1 μM (Fig. 1). Since *B. thetaiotaomicron* is a predominant anaerobe, glucose may serve as an inhibitor for azo reduction in the gastrointestinal tract.

The addition of flavin mononucleotide to the reaction mixture caused a marked linear enhancement of azo reduction by *B. thetaiotaomicron*. Figure 2 exhibits a linearity up to 10 μM with a cell concentration of 5.4 mg/ml (dry weight). The linearity then increased to 20 μM at a cell concentration of 11.0 mg/ml. The following electron carriers stimulate the azo reductions: methyl viologen, benzyl viologen, phenosafranin, neutral red, crystal violet, flavin adenine dinucleotide, menadione, and Janus Green B (Table 2). These data suggest that extracellular electron acceptors can stimulate azo dye reduction.

The toxic effect of amaranth has been reported to be attributable to two metabolites, sodium naphthionate, which causes sternebral

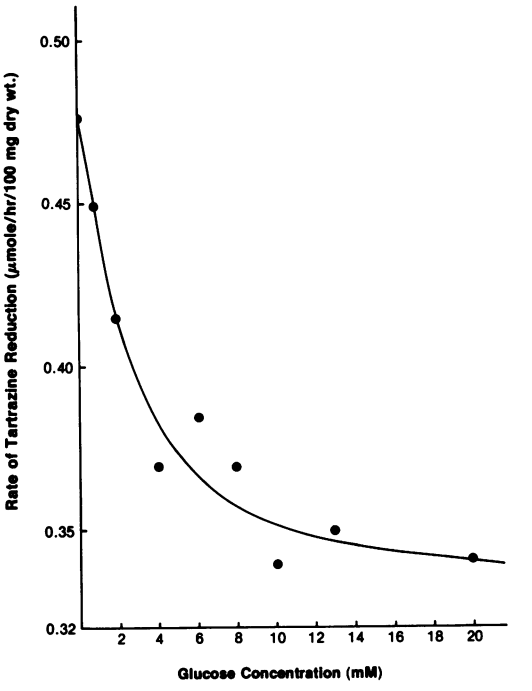

FIG. 1. *Effect of glucose on the rate of tartrazine reduction by B. thetaiotaomicron.*

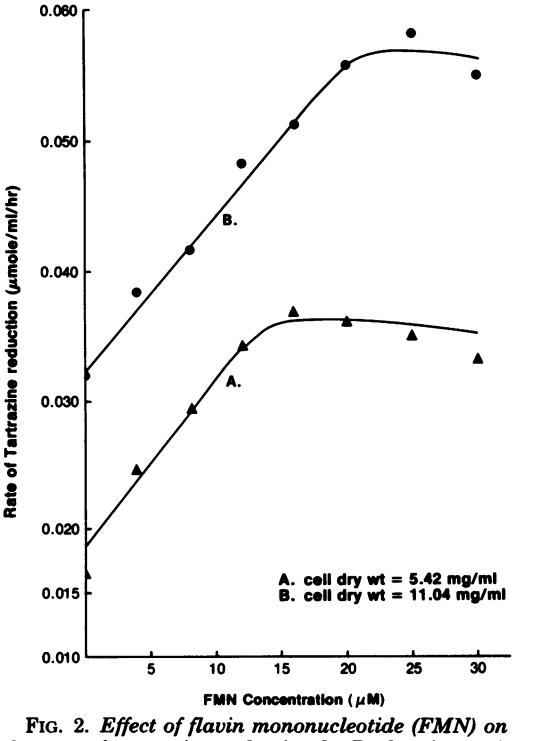

FIG. 2. *Effect of flavin mononucleotide (FMN) on the rate of tartrazine reduction by B. thetaiotaomicron.*

TABLE 2. *Effect of electron carriers on tartrazine reduction of B. thetaiotaomicron (C20-14)*

Cofactor added (5 μM)	Tartrazine reduced (μmol/h per 100 mg [dry wt])
Methyl viologen	0.9
Benzyl viologen	0.9
Crystal violet	0.9
Neutral red	0.9
Phenosafranin	0.7
Flavin adenine dinucleotide	0.7
Menadione	0.6
Janus Green B	0.5
Flavin mononucleotide	0.6
Control	0.2

abnormality in fetuses, and the R-amino salt, which causes skeletal abnormality (10). 1-Amino-2-naphthol, produced by the reduction of Orange II, has been reported to induce bladder tumors (4, 5). Other compounds such as Ponceau SX, Orange II, Allura Red, tartrazine, and methyl orange are all similar in chemical structure. They may all be reduced by intestinal anaerobic bacteria to yield structurally similar products (aromatic amines).

The azo dyes studied are food colorants for food, drugs, or cosmetics, except for methyl orange, which is used as a dye in the textile and printing industries. They are water soluble and are readily ingested in food and water, etc. They are not absorbed in the small intestine and are metabolized by the intestinal bacteria in the colon. The metabolites can be excreted in feces or can be absorbed into the general circulation and excreted in the urine (18). The metabolism of tartrazine in animals, for example, has been studied by several investigators (11, 13). The major urinary metabolite is sulfanilic acid, which is probably produced by the reduction of tartrazine by the intestinal microorganisms (17). Another possible metabolite, sulfophenyl-3-carboxy-4-aminopyrazolone, was not found. Westöö (21) has shown sulfophenyl-3-carboxy-4-aminopyrazolone to be the precursor of the purple pigment, an analog of rubazoic acid, found in the feces of rats given oral doses of tartrazine. In our experiments, bacterial cultures containing reduced tartrazine developed a purple color after exposure to air. This substance had adsorption spectrum maxima at 360 and 540 nm, which were substantially in agreement with the spectrum given by Westöö (21).

The organisms used in this study appear in high numbers in human feces and represent some of the predominant microbes of the human gut (6). The reduction observed occurs anaerobically, and thus the gastrointestinal tract, particularly the colon, which is the most anaerobic environment in the body, is probably the primary site for the reduction of azo dyes.

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