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Reduction of Polymeric Azo and Nitro Dyes by Intestinal Bacteria

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The O_2-sensitive reduction of high-molecular-weight aromatic azo and nitro dyes by intestinal bacteria appears to be mediated by low-molecular-weight electron carriers with $E_0' = -200$ to -350 mV. This process may allow the design of polymeric azo prodrugs for specific release of certain aromatic amines in the colon.

The ability of the intestinal microflora to reduce the azo and nitro groups of various xenobiotic compounds has been known for many years (15, 19). Whereas enzymatic reduction of azo compounds has been demonstrated with hepatic microsomes (12) and cytosol (13), the specific components of the intestinal microflora participating in azo or nitro reduction and the mechanisms of these processes are poorly understood (1, 6, 14). This study was initiated by the finding that intestinal bacteria under anaerobic conditions could reduce high-molecular-weight polymeric derivatives of certain azo dyes at rates comparable with those of the low-molecular-weight parent compounds, despite large differences in the membrane permeabilities of these agents (J. P. Brown, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, I72, p. 123).

The objective of this report is to present data on the reduction of high-molecular-weight polymeric azo and nitro dyes by intestinal bacteria and their enzymic extracts and to evaluate these and other data with respect to the probable mechanism(s) affecting azo and nitro reduction of poorly permeable xenobiotic compounds in the mammalian lower bowel.

(A preliminary report of this study was presented at the XII International Congress of Microbiology, Munich, Germany).

The polymeric azo dyes used in this study are based on the food dyes sunset yellow (FD&C yellow no. 6) and tartrazine (FD&C yellow no. 5) and have been described previously (4, 9). In addition, a similar polymeric derivative of 4-amino-5-nitrobenzene sulfonic acid sodium salt was employed (Fig. 1 and D. J. Dawson, K. M. Otteson, and R. Davis, German patent 2,754,485, 1978) as well as the nonpolymeric parent compounds. The polymers were prepared at Dynapol, and their azo or nitro group contents (equivalents per gram) were determined by titration with titanous chloride or chromous chloride, respectively. The food colors were obtained from Allied Chemical Co. (Morristown, N.J.), and the 4-amino-5-nitrobenzene sulfonate sodium was from Mobay Chemical (Pittsburgh, Pa.). Azo and nitro reduction was observed by spectrophotometric measurement of visible absorption at the λ_{max} value for each substrate dye (Poly S and sunset yellow, 475 nm; Poly T and tartrazine, 430 nm; Poly Y, 423 nm). Measurements were made on supernatants of anaerobic whole-cell suspensions at discrete sampling times, and they were made continuously with sonic cell-free preparations (2) using special anaerobic cuvettes (precision cells, Hicksville, N.Y.; T-27). The ability to reduce polymeric azo dyes was not limited to a particular component(s) of the intestinal microflora, but rather seemed to be shared by all bacteria tested to a varying degree (Table 1). Similar results have been reported for low-molecular-weight azo dyes (3). Also, as reported earlier (Brown, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, I72, p. 123), the reduction of polymeric azo dyes by bacterial cell suspensions almost invariably is stimulated by a variety of low-potential ($E_0' = -200$ to -350 mV) electron carriers (Table 2), particularly flavins, such as FMN. Again, similar observations have been made for low-molecular-weight azo dyes (3).

The similarities demonstrated between azo reduction and nitro reduction, their hepatic and intestinal bacterial catalyses (18) and sensitivity to oxygen (11), and the possibility of their forming free radical intermediates (11) are further extended by the observed bacterial reduction of a polymeric nitro dye such as Poly Y-607 (Table 2). The results obtained with mixed rat cecal bacteria are quite similar to those seen previously with individual bacterial species and high-

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TABLE 1. *Reduction of polymeric azo dyes by cell suspensions of selected anaerobic and facultative bacteria*[a]

Test organism (ATCC)	Poly T		Poly S	
	Control	FMN	Control	FMN
Clostridium ramosum (25582)	1.41[b]	3.81	++[c]	++
Clostridium paraputrificum (17864)	9.28	7.74	+++	+++
Clostridium sporogenes (11437)	4.34	9.18	++	++
Lactobacillus catenaforme (25536)	3.76	4.43	+++	+++
Acidaminococcus fermentans (25085)	0.13	0.80	+	+
Bacteroides fragilis (8482)	1.00	2.93	+	+
Ruminococcus bromii (27255)	0.18	NT[d]	+	+
Veillonella parvula (10790)	(0.14)	(3.5)	+	+
Peptococcus prevotii (9321)	0.52	0.84	++	++
Bifidobacterium adolescentis (15703)	0.59	2.21	+++	+++
Eubacterium aerofaciens (25986)	0.66	1.60	+	+
Salmonella typhimurium LT-2	0.65	2.75	1.57	3.66
Salmonella paratyphi (9150)	0.327	1.42	ND[d]	1.61
Proteus vulgaris (6380)	0.32	0.97	4.13	7.53
Klebsiella pneumoniae (4352)	0.29	0.44	12.2	13.7
Pseudomonas aeruginosa (14207)	0.83	1.25	7.0	10.0
Salmonella typhimurium TA1537	5.9	12.8	3.4	6.76
Streptococcus faecium (19580)	0.49	5.94	3.9	8.78

[a] Anaerobes (*C. ramosum* through *E. aerofaciens*) were grown in 250-ml culture bottles containing prereduced anaerobically sterilized Schaedler's broth (BBL Microbiology Systems, Cockeysville, Md.), and facultative anaerobes were grown unshaken in 250 ml of Trypicase soy broth (BBL) in 500-ml Erlenmeyer flasks. All flasks were inoculated with a sufficient quantity of freshly grown culture (0.1 to 5.0 ml) to give heavy growth in 24 to 30 h at 37°C (early- to mid-stationary phase). The cells were harvested by centrifugation at 25°C (Sorvall RC2B centrifuge) and suspended unwashed in anaerobic VPI diluent (8). The VPI diluent contained (per cent): gelatin, 0.2; cysteine, 0.05; resazurin, 0.001; $CaCl_2$ (anhydrous), 0.01; $MgSO_4 \cdot 7H_2O$, 0.01; K_2HPO_4, 0.05; KH_2PO_4, 0.05; $NaHCO_3$, 0.5; and NaCl, 0.1; diluent pH, 6.8 to 7.1 (see 8 for preparation). Aliquots of buffered cell suspensions (5.0 ml) were then added to assay tubes containing 1.68 μeq of azo bond of polymeric substrate dye and 20 nmol of FMN (Sigma Chemical Co., St. Louis, Mo.) where indicated (3.0 ml, total). Samples of the cell suspensions were centrifuged, washed once with distilled water, transferred to tared aluminum weighing dishes, and dried at 110°C to determine cell concentration. Assay tubes contained 10 to 15 mg of cells (dry weight) per 8.0-ml volume. The tubes were incubated anaerobically at 37°C for 24 h.

[b] Average rate for first 5 h (or 24 h) in nequivalents h^{-1} mg of $cells^{-1}$ (dry weight).

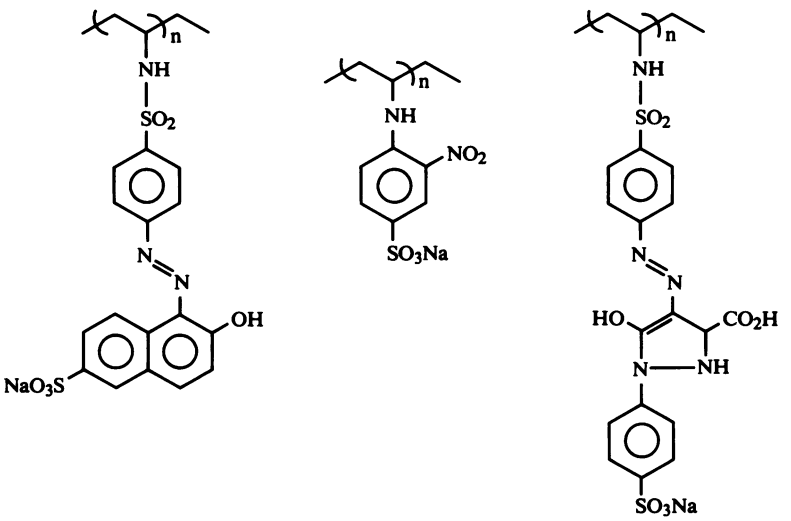
[c] Polymer bound to cells, with the degree of reduction estimated visually: + = slight, ++ = moderate, +++ = nearly complete.

[d] NT, not tested. ND, not determined.

and low-molecular-weight azo dyes (3). In our laboratory, benzyl viologen has proved to be the most effective mediator of both azo and nitro reduction by bacterial cell suspensions.

The reduction of azo and nitro polymers was next investigated in cell-free preparations of rat cecal bacteria. Whereas both azo and nitro reduction could be demonstrated in crude cell-free extracts containing a reduced nicotinamide adenine dinucleotide-generating system and benzyl viologen, the azo-reducing activity was much enhanced by gel filtering these extracts to remove small molecules (Table 3). The reduction of azo dyes in the presence of benzyl viologen was rapid and zero order to very low substrate dye concentrations. This process is oxygen sensitive since the reduced mediator is usually autooxidizable. In addition to this rapid carrier-mediated azo reduction, two other processes were apparent. In the absence of mediator and under aerobic conditions, a reduced nicotinamide adenine dinucleotide-dependent azo reduction proceeds at a very slow rate (~9 nmol h^{-1} ml^{-1} with sunset yellow). Under anaerobic conditions without mediator, both azo and nitro reduction proceed slowly. Findings similar to these have been reported for a number of low-molecular-weight azo dyes with crude cell-free extracts of *Streptococcus faecales* (6), *Fusobacterium* sp. 2 (7), reduced nicotinamide adenine dinucleotide, and flavin mononucleotide (FMN).

In summary, whereas the hypothesis of extracellular reduction of azo and nitro compounds by small molecules has not been proven, most of the key findings can best be explained by such a mechanism acting in concert, perhaps with intracellular "azo and nitro reductases." These findings may be summarized as follows: (i) relatively rapid reduction of high-molecular-weight azo and nitro compounds with low permeability;



Poly S Poly Y-607 Poly T

FIG. 1. *Chemical structures of polymeric azo and nitro dyes. Average number of units per polymer chain, $n \cong 100$.*

TABLE 2. *Effect of exogenous electron mediators on reduction of a polymeric nitro dye by anaerobic suspensions of rat cecal microbes*[a]

Mediator	E_0' (mV)	Reduction at: 4 h %	4 h Rate[b]	24 h %	24 h Rate[b]
None		16	6.8	22	1.5
Methyl viologen	−440	16	6.5	26	1.7
Benzyl viologen	−339	93	38	96	6.6
Neutral red	−325	50	21	70	4.8
Phenosafranin	−252	23	9.4	50	3.4
FMN	−219	16	6.4	41	2.8
Anthraquinone 2,6-disulfonate	−185	16	6.5	24	1.6
Indigotine	−125	15	6.2	18	1.3

[a] Assays were carried out in anaerobic test tubes, 8.0-ml total volume, containing 1.74 μeq of NO_2 of poly Y-607, 10 nmol of respective mediators; 10 to 15 mg of cecal microorganisms (dry weight) obtained by suspending fresh cecal contents in anaerobic VPI diluent (1 g/10 ml) and filtering through glass wool, 88 μmol of α-D-glucose, 1.25 mmol of potassium phosphate buffer (pH 7.4), 1.25 mg of dithiothreitol (Cleland's reagent), dilute Krebs Ringer salts (NaCl, 79 nmol; KCl, 7.1 nmol; $CaCl_2$, 5.4 nmol; $MgSO_4$, 1.8 nmol), and an N_2 (O_2-free) gas phase. The sources of mediators were as follows: phenosafranin and indogotine, Allied Chemical Co.; methyl viologen and FMN, Sigma Chemical Co., St. Louis, Mo.; neutral red and anthraquinone 2,6-disulfonic acid disodium salt, Matheson, Coleman & Bell, Los Angeles, Calif.; and benzyl viologen, K&K Laboratories Division, ICN Pharmaceuticals, Inc., Plainview, N.Y.

[b] Average reduction rate in nanoequivalents h^{-1} mg cells^{-1} (dry weight).

TABLE 3. *Anaerobic reduction of azo and nitro dyes by Bio Gel P100-filtered fractions of cell-free extracts of rat cecal bacteria*[a]

Substrate dye	Concn	Reduction (μmol or μeq/h per ml of fraction): Control	Benzyl viologen (53 μM)
Sunset yellow	25 μM	0.98	49.1
Tartrazine	25 μM	0.19	31.2
Poly S	20 μeq/liter	0.69	81.2
Poly T	20 μeq/liter	0.79	97.0
Poly Y-607	25 μeq/liter	0.012	0.099

[a] Rat cecal contents were suspended (1 g [fresh weight] per 10 ml) in VPI diluent, filtered through glass wool, and sonically disrupted (at 50W for 10 min, at 4–8°C). Cell-free extract was prepared by centrifugation at 13,000 × *g* for 25 min at 4°C. A 10-ml amount of cell-free extract was applied to a column (30 by 210 mm) of Bio-Gel P100 (Bio-Rad) equilibrated with 0.01 M potassium phosphate buffer, pH 7.40. A 10-ml amount of a 280-nm-absorbing exclusion peak was collected for azo and nitro reduction assays. Assay volumes (3.0 ml each) contained 0.06 to 0.075 μmol or μeq of substrate dye, 125 μmol of potassium phosphate buffer (pH 7.40), 2.5 μmol of glucose-6-phosphate sodium, 0.75 μmol of oxidized nicotinamide adenine dinucleotide phosphate, 3 U of glucose-6-phosphate dehydrogenase, and 0.4 μmol of benzyl viologen (side arm). The assay was carried out in a special anaerobic cuvette (Precision Cells) gassed with N_2 (O_2 free).

(ii) relatively little structure and activity correlation with respect to the reduction of azo dyes by intestinal bacteria (10, 16, 17); (iii) stimulation of azo and nitro reduction by bacterial cells with a variety of exogenous redox mediators (E_0' = −200 to −350 mV); (iv) demonstrated reduction of such mediators by bacterial enzymes and coupling of reduced mediators with nonenzy-

matic reduction of substrate dyes in vitro (6); and (v) demonstrated reduction of polymeric azo dyes in vivo (rats) indicating the presence of natural mediators or extracellular reducing enzymes or both (9).

This latter point is the basis for the design of a new class of site-specific controlled-release polymeric prodrugs for the therapy of lower bowel and possibly other diseases (T. M. Parkinson, J. P. Brown, R. E. Wingard, Jr., U.S. patent 4,190,176, 26 Feb. 1980).

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