Evidence for Direct-acting Oxidative Genotoxicity by Reduction Products of Azo Dyes

Elizabeth A. Sweeney,1 J. Kevin Chipman,2 and Stephen J. Forsythe1

1Department of Life Sciences, The Nottingham Trent University, Nottingham, England; 2School of Biochemistry, The University of Birmingham, Birmingham, England

The intestinal flora forms a complex ecosystem that metabolizes dietary and endogenous nutrients under primarily anaerobic conditions. The ingestion of azo dyes has been proposed as one source of potential genotoxic agents. Many intestinal bacteria are able to reduce the azo bond (termed azofission), which liberates the substituted naphthol compounds. The standard Ames test has not demonstrated mutagenicity either by various common food colorings or by their reduced end products in Salmonella typhimurium strains TA98 and TA100. In contrast, genetic toxicity was demonstrated in the Escherichia coli differential kill assay and in S. typhimurium TA102 for the reduced dyes. The superoxide free radical was produced by the azo dyes only after reduction by the intestinal bacteria Enterococcus faecalis and Bacteroides thetaiotaomicron. — Environ Health Perspect 102(Suppl 6):119–122 (1994)

Key words: azo dyes, Salmonella typhimurium TA102, superoxide radicals,azo reduction, oxidative mutagens, genotoxicity

Introduction

Azo compounds are the most common synthetic colorings used in the food, pharmaceutical, and cosmetic industry. Also known as coal tar dyes, they contain an aromatic ring linked by an azo bond to a second naphthalene or benzene ring. Coloring matter entering the intestinal tract is subjected to the action of acid, digestive enzymes, and microflora. Azo compounds may reach the intestine directly after oral ingestion or through the bile after parenteral administration. They are reduced by azo reductases from intestinal bacteria and, to a lesser extent, by enzymes of the cytosolic and microsomal fractions of the liver. The first catabolic step in the reduction of azo dyes, which is accompanied by a decrease in the visible light absorbance and then decolorization of the dye, is the reduction of the azo bond to produce aromatic amines (Figure 1). Aromatic amines, some of which are known carcinogens, have been found in the urine of dyestuff workers and test animals following administration of azo dyes (1). Although a number of commonly used dyes are not mutagenic in Salmonella typhimurium strains TA98 and TA100 even after azo-reduction, the production of reactive oxygen species from \( \text{\(\cdot\)}\)hydroxy aromatic amine products has been suggested (2,3). This article reports the generation of superoxide anions from reduced azo dyes and aminonaphthols and the genetic toxicity of these products in Escherichia coli and a Salmonella strain (TA102), which is sensitive to oxidants.

Materials and Methods

Bacterial Strains

The organisms studied were obtained from The Nottingham Trent University Culture Collection (England), except S. typhimurium strains, which were obtained from B. Ames, University of California, Berkeley. Enterococcus faecalis was grown in tryptone soya broth, and Bacteroides thetaiotaomicron was grown anaerobically in Schaedlers broth; both were incubated at 37°C for 24 hr. Cells were harvested by centrifugation at 7890 g for 10 min and anaerobically washed once with 0.5 M potassium phosphate buffer, pH 7.4. The bacterial pellets were resuspended to one-twentieth of their previous volume in phosphate buffer and immediately used for azo reduction.

Figure 1. Possible reduction mechanism for sunset yellow.

This paper was presented at the Fifth International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds held 18-21 October 1992 in Würzburg, Germany.

E.A. Sweeney is grateful for financial support from The Nottingham Trent University.

Address correspondence to E. A. Sweeney, Department of Life Sciences, The Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK. Telephone 0602 419418. Fax 0602 488636

Environmental Health Perspectives 119
Materials

Sunset yellow (Colour Index [C.I.] 15985) and carmoisine (C.I. 14720) were obtained from Gurr, BDH Chemicals Ltd (Poole, Dorset, UK), Amaranth (C.I. 16185) and all other chemicals used were obtained from Sigma Chemical Co. (Poole, Dorset, UK). It should be noted that purity of the azo dyes was 85%. The electron carriers used were flavin mononucleotide (FMN), riboflavin (RF), and flavin adenine dinucleotide (FAD).

Bacterial Reduction of Azo Dyes

The standard assay mix consisted of 4.2 ml degassed potassium phosphate buffer (50 mM, pH 7.4), 0.4 ml washed cell suspension with or without glucose (0.2 ml, 10% wt/vol) and electron carriers in narrow assay tubes (18 × 142 mm). The various electron carriers were prepared anaerobically at 0.025 mM. The reaction mix was incubated at 37°C prior to starting the reaction by the addition of 0.1 ml of the appropriate azo dye (10 mM). Decoloration rate was determined spectrophotometrically at the wavelength of the maximal absorbance of the azo dye.

Chemical Reduction Assay

Dyes were reduced either with sodium dithionite according to the method of Zbaida and Levine (4), or with stannous chloride as described by Gasparic (5).

Detection of Superoxide Anion Radical

Superoxide anion (O$_2^-$) production was detected by the reduction of nitroblue tetrazolium (NBT) and its inhibition by superoxide dismutase (SOD), according to the method of Oberley et al. (6). The sample (100 μl) to be tested was added to 0.8 ml potassium phosphate buffer (50 mM, pH 7.8) containing 0.056 mM NBT, 0.1 mM EDTA, 0.1 mM xanthine, 0.06% wt/vol Triton X100, and 0.33 mg/ml gelatin, ± 100 μl SOD. Immediately after mixing, the absorbance at 560 nm was measured with respect to time. SOD activity was assayed using a xanthine/xanthine oxidase technique (6). SOD was inactivated by boiling for 15 min.

Differential Killing Assay

The E. coli differential cytotoxicity assay was used to determine the genotoxic activity of the azo dyes according to the method described by Tweats et al. (7). The tester strains were E. coli WP2, (repair proficient) and E. coli CM871 (repair deficient). E. coli CM871 is a uvrA recA lexA triple mutant that combines extreme repair deficiency with near wild-type growth. Compounds were accepted as genotoxic if survival of the repair deficient strain was at least 4-fold lower than survival of the repair proficient strain.

All compounds were tested for genotoxicity with and without prior incubation with either E. faecalis or B. thetaiotaomicron cell suspensions. Incubation was for 1 hr with E. faecalis or overnight with B. thetaiotaomicron. Final concentrations of agents used were azo dyes, 5 mM; 1-aminonaphthol HCl, 0.1 mM; 1-aminonaphthol-4-sulfonic acid, 10 mM; 4-aminonaphthalene HCl, 1 mM; and 4-aminonaphthalene-sulfonic acid sodium salt, 5 mM.

Genotoxic activity was assessed after removing cells by centrifugation in a microfuge (8000g, 5 min), followed by filtration (0.2 μm Dynaguard filters, New Brunswick). An aliquot (100 μl) of the filtrate was added to the diluted E. coli strain test (200 μl), and the genotoxic activity was determined. Inhibition by UV was used as a positive control for CM871.

The Rapid Automated Bacterial Impedance Technique (RABIT), (Don Whiteley Scientific Ltd, Shipley, UK) and plate counts were used to measure microbial survival (8).

Salmonella Mutagenicity Test

The protocol used was essentially that of Ames et al. (9). The strains of S. typhimurium used were TA98, TA100, and TA102. Liver postmitochondrial supernatant was not incorporated in this test. Bleomycin was used as a positive control for TA102 (9).

Results

The reduction of the three dyes by E. faecalis and B. thetaiotaomicron is summarized in Table 1. Azo reductase activity was stimulated by the addition of extracellular electron acceptors such as FMN, FAD, and RF. The presence of glucose on reduction of azo dyes was inhibitory for B. thetaiotaomicron, but not for E. faecalis. This may be because of the different electron transport systems of these organisms.

After incubation with E. faecalis, the cleavage products of sunset yellow and amaranth were found to be genotoxic in the bacterial differential assay (Figure 2). Carmoisine was marginally genotoxic after azo reduction, but survival of E. coli CM871 was not consistently 4-fold less than survival of E. coli WP2. Similar results were obtained on incubation with B. thetaiotaomicron. Unreduced dyes were not genotoxic in this assay. Of the four coupling components tested, 1-amino-2-naphthol HCl and 1-amino-2-naphthol-4-sulfonate are considered genotoxic.

Mutagenicity studies on the same samples using the standard Ames test with S. typhimurium TA98 and TA100 showed no mutagenicity. When compounds were retested using S. typhimurium TA102, reduced amaranth and reduced sunset yellow gave a doubling of the spontaneous reversion rate (Figure 3). This strain detects a variety of oxidants as mutagens that are not detected by strains TA98 or TA100 (10).

Generation of O$_2^-$ was observed for reduced dyes and some aminonaphthols but not for unreduced dyes (Figures 4, 5). The increase in absorbance at 540 nm (NBT assay) was inhibited by SOD, while inactivated SOD showed no inhibition. Table 2 summarizes the rates of O$_2^-$ production from unreduced and reduced azo dyes and their coupling components. Results were further confirmed by an ability to reduce cytochrome c (data not shown). Negligible

Table 1. Cofactor requirements for azoreductase activity.*

| Compound       | Cofactor | Rate of decoloration, μmole/min/mg cell dry wt |
|----------------|----------|-----------------------------------------------|
|                |          | by E. faecalis | by B. thetaiotaomicron |
| Amaranth       | –        | NQ            | NQ                      |
| Amaranth       | GI       | 40.7 ± 2.4    | NQ                      |
| Amaranth       | FMN      | NQ            | 7.2 ± 1.24              |
| Amaranth       | GI + FMN | 113.5 ± 7.04  | 3.9 ± 0.63              |
| Amaranth       | GI + FAD | 83.6 ± 3.5    | 5.5 ± 0.36              |
| Amaranth       | GI + RF  | 75.6 ± 5.36   | 4.6 ± 0.17              |
| Sunset yellow  | –        | NQ            | NQ                      |
| Sunset yellow  | GI       | 79.0 ± 1.5    | NQ                      |
| Sunset yellow  | FMN      | 3.3 ± 0.17    | 7.5 ± 0.21              |
| Sunset yellow  | GI + FMN | 123.5 ± 11.1  | 5.4 ± 0.25              |
| Sunset yellow  | GI + FAD | 93.5 ± 1.98   | 6.3 ± 0.25              |
| Sunset yellow  | GI + RF  | 78.1 ± 3.6    | 4.6 ± 0.48              |

Abbreviations: NQ, nonquantifiable (rate less than 1 nm dye reduced per minute per milligram cell dry weight); GI, glucose; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide. *Mean ± SD of three independent experiments. **Cell dry weight = 0.76 mg/ml. ***Cell dry weight = 0.51 mg/ml.
O$_2^-$ was produced by 4-amino napthalene and its sodium salt.

**Discussion**

It has been demonstrated that there are no cytochromes present in cell-free extracts of *E. faecalis* and that the transfer of electrons from reduced pyridine nucleotides to electron acceptors was mediated by a flavoprotein (F1).

In view of their charged nature, it is uncertain whether FAD and FMN could readily shuttle across bacterial cell walls and membranes under physiologic conditions. In addition, it is unlikely that highly charged sulfonated dyes could pass through the cell wall of these organisms. An electron shuttle could affect the reduction of the dyes remaining extracellular. It may also serve as a partial explanation for the oxygen sensitivity of these reduction reactions, reduced flavin having a greater affinity for oxygen than it has for the azo dye.

In this article, we have tested three azo dyes for genotoxicity following bacterial reduction of the dye. Both amaranth and sunset yellow, when reduced, induced cytotoxicity indicating DNA damage in repair-deficient *E. coli* in the absence of hepatic enzymes, but they failed to mutate *S. typhimurium* strains TA98 and TA100. In contrast, strain TA102, which detects oxidative mutagens, (10,12) was mutated by reduced amaranth and reduced sunset yellow.

The findings suggest that various azo dye products are genotoxic, not through N-hydroxylation and esterification, which is characteristic of many aromatic amines (2,13), but rather through a mechanism involving oxygen radicals (3,14). In further support, the production of O$_2$ radical from reduced azo dyes was detected by the

| Compound                      | Rate of O$_2$ production, µmole compound/min |
|-------------------------------|---------------------------------------------|
| Xanthine                      | 0.014 ± 0.0021                              |
| Amaranth                      | 0                                           |
| Carmoisine                    | 0                                           |
| Sunset yellow                 | 0                                           |
| Amaranth$^b$                  | 0.023 ± 0.0017                              |
| Carmoisine$^c$                | 0.018 ± 0.0004                              |
| Sunset yellow$^d$             | 0.026 ± 0.0011                              |
| Amaranth$^e$                  | 0.062 ± 0.0018                              |
| Carmoisine$^f$                | 0.0188 ± 0.0034                             |
| Sunset yellow$^g$             | 0.055 ± 0.0023                              |
| 1-Amino-2-naphthol HCl        | 1.066 ± 0.0045                              |
| 4-Amino-2-naphthol sulfonic acid & HCl | 0.022 ± 0.0026 |
| 4-Aminobiphthalene HCl        | 1.336 × 10$^{-4}$ ± 0.3 × 10$^{-5}$        |
| sulfonic acid, Na salt        | 0.033 × 10$^{-4}$ ± 0.4 × 10$^{-5}$        |

$^a$ Mean ± SD of three independent exponents.

$^b$ Reduced by *E. faecalis.*

$^c$ Reduced by NaCl.

---

Figure 2. Bacterial genotoxicity of azo dyes using bacterial differential assay. A, amaranth; SY, sunset yellow; C, carmoisine; 1) 1-amino-2-naphthol hydrogen chloride; 2) 1-amino-2-naphthol-4-sulfonic acid; 3) 4-amino naphthalene sulfonic acid, sodium salt.

Figure 3. Bacterial mutagenicity of azo dyes, Ames test, and *S. typhimurium* TA102. A, Amaranth; SY, sunset yellow; C, carmoisine; 1) 1-amino-2-naphthol hydrogen chloride; 2) 1-amino-2-naphthol-4-sulfonic acid; 3) 4-amino napthalene sulfonic acid, sodium salt. The mean number of viable bacteria and plate for spontaneous and control (1 mg/ml) incubations were 140 and 1050.

Figure 4. Superoxide production from sunset yellow reduced by *E. faecalis.*

Figure 5. Superoxide production from sunset yellow reduced by stannous chloride.

Figure 6. Possible mechanism for production of superoxide anions from reduced sunset yellow. Adapted from Nakayama (3).
reduction of nitrotetrazolium blue and confirmed using cytochrome c. The mechanism by which these species are formed is not clear, but they may be specific for o-hydroxy aromatic amines and involve the reactions postulated by Nakayama (3) and summarized in Figure 6. There may be a requirement for iron, as in Fenton chemistry, where \( \text{O}_2^{-} \) is converted via \( \text{H}_2\text{O}_2 \) to the highly reactive OH species that is known to damage DNA (14). All of the compounds shown to generate active oxygen species have a hydroxyl substituent ortho to the amino function. However, this does not explain the weakly mutagenic results for carmoisine. \( \text{O}_2^{-} \) was detected for 1-amino-2-naphthol HCl and 1-amino-2-naphthol sulfonate, while no active oxygen was detected for 4-aminonaphthalene HCl and its sodium salt.

We are currently studying the ability of o-hydroxy aromatic amines to produce deoxyribose oxidation in DNA. The identification of this type of DNA damage hitherto not detected by many conventional genotoxicity assays may have important implications regarding the continued use of azo dyes in foods.

REFERENCES

1. Cerniglia CE, Zhuo Z, Manning BW, Federle TW, Helfich RH. Mutagenic activation of the benzidine-based dye Direct Black 38 by human intestinal microflora. Mutat Res 175:11–16 (1986).
2. Kimura T, Kodama M, Nagata C. Nitrooxide radicals generated from carcinogenic aminoazo dyes during their metabolism in vivo and in enzymatic systems in vitro. Biochem Pharmacol 28:557–560 (1979).
3. Nakayama T, Kimura T, Kodama M, Nagata C. Generation of hydrogen peroxide and superoxide anions from active metabolites of naphthalamines and aminoazo dyes: its possible role in carcinogenesis. Carcinogenesis 4:765–769 (1983).
4. Zbaida S, Levine WG. Role of electronic factors in binding and reduction of azo dyes by hepatic microsomes. J Pharmacol Exp Ther 260:554–561 (1992).
5. Gasparic J. Chemical degradation methods. In: The Analytical Chemistry of Synthetic Dyes (Venkataraman K, ed). New York:John Wiley and Sons, 1977:299–316.
6. Oberley LW, Spitz DR. Assay of superoxide dismutase activity in tumour tissue. In: Methods in Enzymology, Vol 105 (Packer L, ed). London:Academic Press, 1984:457–464.
7. Tweets D, Green M, Muriel WJ. A differential kill assay for mutagens and carcinogens based on an approved repair deficient strain of Escherichia coli. Carcinogenesis 2:189–195 (1981).
8. Forsythe SJ. The rapid detection of direct-acting DNA mutagens by electrical impedance with DNA repair deficient strains of Escherichia coli. Letts Appl Microbiol 11:30–32 (1990).
9. Ames BN, McCann J, Yamazaki E. Methods for detecting carcinogens and mutagens with Salmonella/mammalian microsome mutagenicity test. Mutat Res 31:347–364 (1975).
10. Levine D, Hollstein M, Christman M., Schwiers E, Ames BN. A new Salmonella tester strain (TA102) with A-T base pairs at the site of mutation detects oxidative mutagens. Proc Natl Acad Sci USA 79:7445–7449 (1982).
11. Gingell R, Walker R. Mechanisms of azo reduction by Streptococcus faecalis II. The role of soluble flavins. Xenobiotica 1:231–239 (1971).
12. DeFlora S, Camoirano A, Zanacchi C. Mutagenicity testing with TA97 and TA102 with 30 DNA damaging compounds, negative with other Salmonella strains. Mutat Res 134:159–165 (1984).
13. Farr SP, Kogoma T. Oxidative stress responses in Escherichia coli and Salmonella typhimurium. Microbiol Rev 55:561–585 (1991).
14. Basaga HS. Biochemical aspects of free radicals. Biochem Cell Biol 68:989–998 (1989).