APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1981, p. 641-648
0099-2240/81/100641-08$02.00/0
Vol. 42, No. 4

Mutagenicity Testing of Some Commonly Used Dyes

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Received 27 March 1981/Accepted 15 July 1981

Seventeen commonly used dyes and 16 of their metabolites or derivatives were tested in the *Salmonella*-mammalian microsome mutagenicity test. Mutagens active with and without added Aroclor-induced rat liver microsome preparations (S9) were 3-aminopyrene, lithol red, methylene blue (USP), methyl yellow, neutral red, and phenol red. Those mutagenic only with S9 activation were 4-aminopyrazolone, 2,4-dimethylaniline, *N,N*-dimethyl-*p*-phenylenediamine, methyl red, and 4-phenyl-azo-1-naphthylamine. Orange II was mutagenic only without added S9. Nonmutagenic azo dyes were allura red, amaranth, ponceau R, ponceau SX, sunset yellow, and tartrazine. Miscellaneous dyes not mutagenic were methyl green, methyl violet 2B, and nigrosin. Metabolites of the azo dyes that were not mutagenic were 1-amino-2-naphthol hydrochloride, aniline, anthranilic acid, cresidine salt, pyrazolone T, R-amino salt (1-amino-2-naphthol-3,6-disulfonic disodium salt), R-salt, Schaeffer's salt (2-naphthol-6-sulfonic acid, sodium salt), sodium naphthionate, sulfanilamide, and sulfanilic acid. 4-Amino-1-naphthalenesulfonic acid sodium salt was also not mutagenic. *Fusobacterium* sp. 2 could reductively cleave methyl yellow to *N,N*-dimethyl-*p*-phenylenediamine which was then activated to a mutagen.

A wide variety of dyes are used in the food, textile, and printing industries and in chemical and biological laboratories (7). For example, trypan blue (3,3'-[3,3'-dimethyl-4,4'-biphenylene)bis(azo)]bis(5-amino-4-hydroxy-2,7-naphthalene disulfonic acid) tetrasodium salt and neutral red (3-amino-7-dimethylaminophenazine hydrochloride) are widely used for marking and staining cells (11, 17). Dyes, whether used as laboratory stains or food, fabric, and paper colorants, are important environmental chemicals.

Many of the water-soluble azo dyes are known to be degraded by intestinal microorganisms in vitro (6) and in vivo (12, 14, 19). The mutagenicity of the dyes and their degradation products might indicate a hazard to workers and consumers who came in contact with these compounds.

We report here the results of mutagenicity testing of some dyes and their metabolites using the *Salmonella*-microsome mutagenicity test developed by Ames et al. (1).

MATERIALS AND METHODS

Ponceau SX, lithol red, ponceau R, allura red, sodium naphthionate, pyrazolone T, R-salt, Schaeffer's salt, and cresidine salt were kindly provided by Adrian B. Leatherman of the Food and Drug Administration, Washington, D.C. The R-amino salt was a gift of W. Link and J. Dantzman of the Division of Color Technology, Department of Health and Human Services. Other compounds used were: amaranth, phenol red, methylene blue (USP), aniline, and sulfanilic acid (Fisher Scientific Co., Fair Lawn, N.J.); sunset yellow, methyl red, methyl yellow, methyl orange, and neutral red (Matheson, Coleman and Bell, Norwood, Ohio); tartrazine, 4-aminopyrazolone, and *N,N*-dimethyl-*p*-phenylenediamine (ICN Pharmaceuticals, Inc., New York, N.Y.); orange II, 4-phenyl-azo-1-naphthylamine, 2,4-dimethylaniline, 1-amino-2-naphthol hydrochloride, 4-amino-1-naphthalene sulfonic acid (sodium salt), sulfanilamide, and anthranilic acid (Eastman Organic Chemicals, Rochester, N.Y.); methyl violet 2B (Hartman-Leddon Company, Philadelphia, Pa.); nigrosin and methyl green (Allied Chemical Corp., New York, N.Y.); and 3-aminopyrene (Sigma Chemical Co., St. Louis, Mo.). The chemical structures of these compounds are shown in Fig. 1 and 2. The sodium azide, 2-nitrofluorene, and 2-aminoanthracene were from the National Cancer Institute's Chemical Respository (IIT Research Institute, Chicago, Ill.), and Aroclor 1254 was from Analabs, New Haven, Conn.

Fusobacterium sp. 2 (6) was inoculated into 500 ml of brain heart infusion broth in a 1-liter round-bottom flask and incubated statically at 37°C for 17 to 19 h. The culture was then centrifuged at 20,000 × *g* for 20 min and washed once with 0.4 M potassium phosphate buffer solution (pH 7.4). Four milliliters of this suspension was added to 4 ml of the phosphate buffer and 2 ml of the dye (2 to 5 μmol/ml). The mixture was incubated anaerobically in a 37°C water bath with

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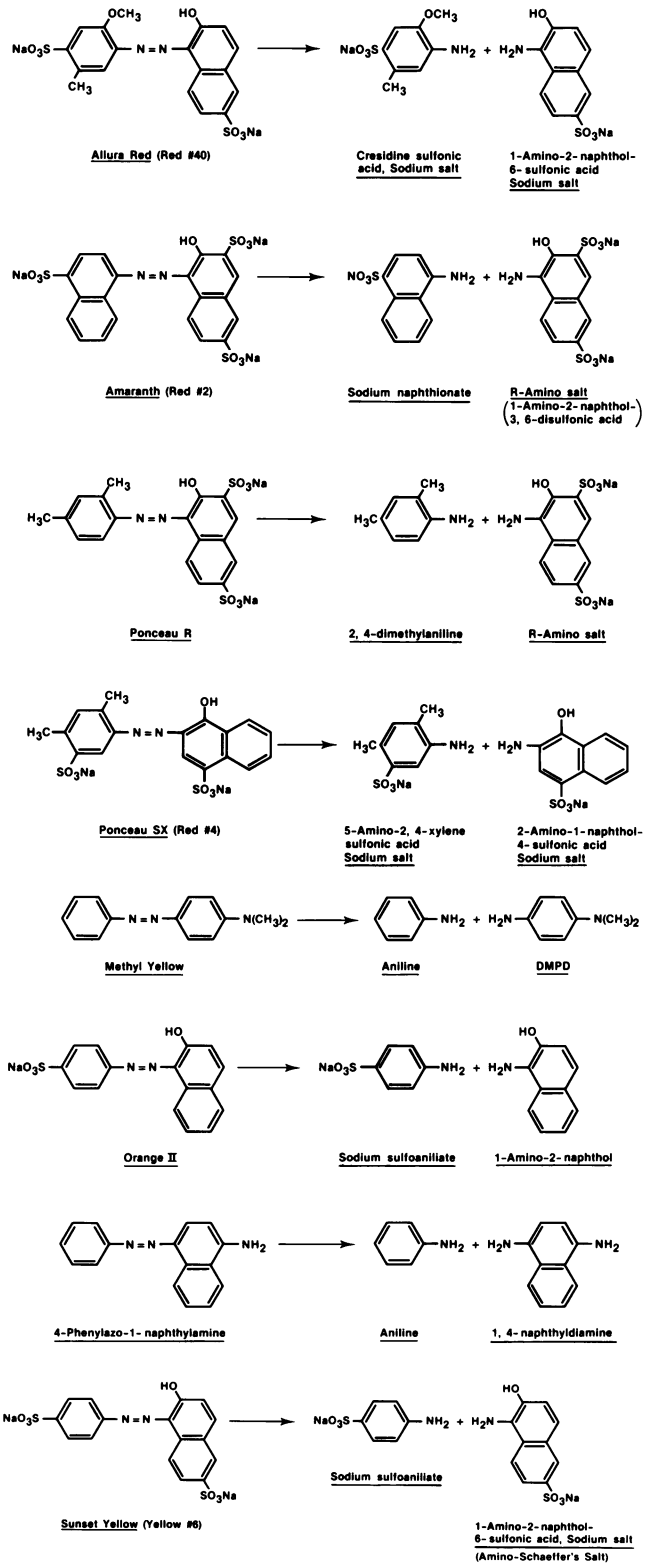
Allura Red (Red #40)
Cresidine sulfonic acid, Sodium salt
1-Amino-2-naphthol-6-sulfonic acid Sodium salt
Amaranth (Red #2)
Sodium naphthionate
R-Amino salt (1-Amino-2-naphthol-3, 6-disulfonic acid)
Ponceau R
2, 4-dimethylaniline
R-Amino salt
Ponceau SX (Red #4)
5-Amino-2, 4-xylene sulfonic acid Sodium salt
2-Amino-1-naphthol-4-sulfonic acid Sodium salt
Methyl Yellow
Aniline
DMPD
Orange II
Sodium sulfoanilate
1-Amino-2-naphthol
4-Phenylazo-1-naphthylamine
Aniline
1, 4-naphthyldiamine
Sunset Yellow (Yellow #6)
Sodium sulfoanilate
1-Amino-2-naphthol-6-sulfonic acid, Sodium salt (Amino-Schaeffer's Salt)

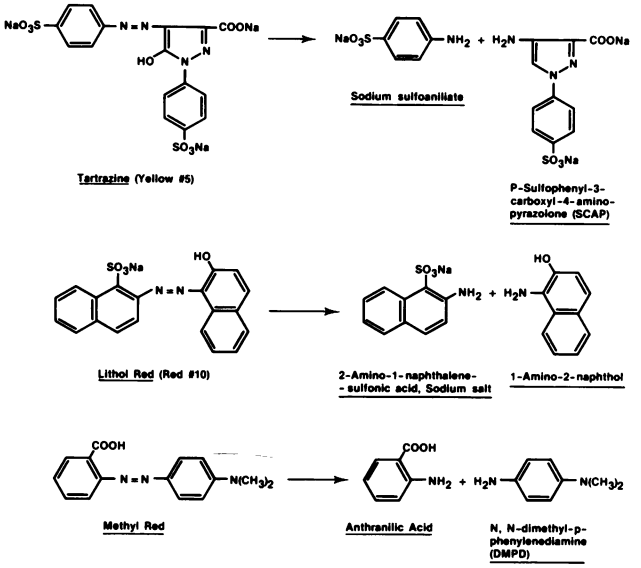

FIG. 1. *Chemical structures of azo dyes and their metabolites.*

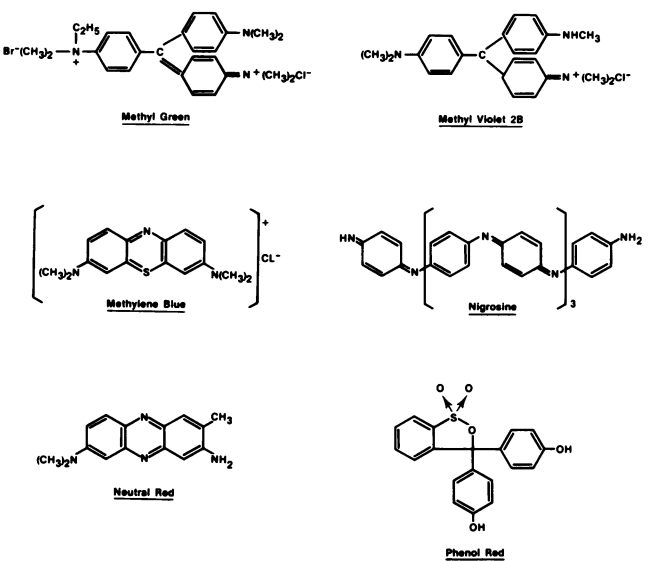

FIG. 2. *Chemical structure of some miscellaneous dyes.*

slow shaking for 1 h. This suspension was then centrifuged at 7,000 × *g* for 20 min. To further purify the supernatant, it was drawn through an ultrafine fritted glass disk filter. The filtrate was screened for mutagenicity in the Ames assay.

Mutagenesis assay. The *Salmonella typhimurium* tester strains TA1535, TA1537, TA1538, TA98, and TA100 were grown in nutrient broth shaken for 14 h at 37°C. The plate incorporation assays were performed as described by Ames et al. (1) with the modifications of Andrews et al. (2). The liquid preincubation assays (1, 21) were timed for 30 min at 37°C in a Dri-block. The revertant colonies were counted by using a hand-held tally. Dimethyl sulfoxide was the solvent, except sterile distilled water was the solvent when sulfanilic acid was used. Liver homogenates (S9) were prepared from male Sprague-Dawley rats stimulated with Aroclor 1254 (500 mg/kg intraperitoneally 5 days before sacrifice). The S9 mix, added in samples of 0.5 ml per plate, contained 3 mg of protein, determined by the method of Lowry et al. (16). The positive control chemicals sodium azide, 9-aminoacridine, 2-nitrofluorene, and 2-aminoanthracene were used with the tester strains. The dose-response curves for the

mutagenic compounds used the tester strain which showed maximum mutagenicity and covered a range of 1 to 1,000 or 5 to 5,000 μg. Mutagenic compounds were assayed by using duplicate plates in at least two independent dose-response curves; the mean values of representative curves are used in the table. A compound was considered mutagenic when the number of revertants above background was at least twice the value of the historical control mean or twice the value of the current control mean, whichever was greater, and a dose-response curve could be demonstrated (22).

Measurement of liver S9 azoreductase. The reaction mixture contained the following components: 0.2 ml of dye (1.25 mM in undiluted dimethyl sulfoxide), 0.3 ml of S9 preparation (45 μl of S9 fraction), 0.2 ml of 0.05 M glucose 6-phosphate, 0.1 ml of 0.01 M reduced nicotinamide adenine dinucleotide phosphate, 0.2 ml of 0.1 M potassium phosphate buffer (pH 7.5), 0.06 ml of 0.1 M $MgCl_2$, and 0.94 ml of deionized water. The reaction was carried out in a 37°C water bath with slow shaking for 5 min.

For methyl red and methyl orange, the reaction was stopped by the addition of 2 ml of 15% trichloroacetic acid, and the mixture was centrifuged at 29,000 × *g* for 20 min. The absorbance of the supernatants was measured at 505 and 510 nm, respectively, with a Beckman DB-GT spectrophotometer. For methyl yellow, the reaction was stopped by the addition of 2 ml of 10 N NaOH, and the mixture was extracted twice with 3 ml of toluene. The absorbance of the toluene extract was measured at 414 nm.

Thin-layer chromatographic analysis and purification of compounds. Thin-layer chromatographic analyses were made on 20- by 20-cm Silica Gel G 250 cellulose plates (Analtech, Inc., Neward, Del.) developed in one or more of the following solvent systems (by volume): methanol-chloroform (7:6); methanol-chloroform (7:3); methanol-chloroform-ammonium hydroxide (6:3:1); methanol-chloroform-acetic acid (6:3:1); tertiary butanol-absolute ethanol (1:1); and 1% NaCl in water and visualized by Ehrlich, ninhydrin, or concentrated H_2SO_4 reagents. Metabolites were identified from their R_f value, color, or ultraviolet absorption on the plates (or a combination of these).

Methyl red, methyl green, methylene blue (USP), neutral red, 4-aminopyrazolone, 3-aminopyrene, and *N,N*-dimethyl-*p*-phenylenediamine were purified by thin-layer chromatography with 20- by 20-cm Silica Gel G 500 plates loaded with large amounts of the compound and developed in the solvent system determined most efficient by prior analyses. Isolated visible bands which appeared were scraped from the plates, and the materials were eluted several times in ~10 ml of warm methanol (40 to 50 ml, total volume). After each elution, the materials were filtered through ultrafine fritted glass, and the eluates were pooled. The methanol was evaporated to dryness under vacuum on a rotary flash evaporator (Buchler Instruments, Fort Lee, N.J.), and the residue was dissolved in a small amount of methanol. This solution was transferred to a test tube, and the methanol was evaporated under a stream of N_2. The remaining material was dissolved in a small volume of methanol and rechromatographed. The compound was considered pure if it gave a single spot corresponding to the band from which it was isolated with the R_f value, color, or ultraviolet absorption of the known pure compound (or a combination of these).

RESULTS

Table 1 shows the compounds and the maximum nontoxic doses tested that were not mutagenic in the tester strains with or without male rat Aroclor S9 mix.

The test compounds that were mutagenic reverted strains TA1538, TA98, and TA100 to histidine prototrophy. Results representative of one of several tests of the positive control chemicals using these three tester strains are shown in Table 2.

1-Amino-2-naphthol hydrochloride and methyl violet 2B were particularly toxic to the bacteria. Ponceau R and R-amino salt were minimally mutagenic without metabolic activation in several tests, but did not meet the criteria for mutagenesis when all of the data were evaluated.

Table 3 shows the historical mean values and standard deviations for the normal background

TABLE 1. *Maximum nontoxic dose tested that was nonmutagenic in TA1535, TA1537, TA1538, TA98, or TA100 with and without male rat Aroclor S9 mix*

Test compound	Maximum nontoxic dose (μg)	
	Plate incorporation test	Liquid preincubation test
Azo dyes		
Allura red	500	500
Amaranth	1,000	500
Ponceau R	5,000	1,000
Ponceau SX	5,000	500
Sunset yellow	5,000	500
Tartrazine	5,000	500
Metabolites or derivatives of azo dyes		
4-Amino-1-naphthalenesulfonic acid, Na salt	5,000	500
1-Amino-2-naphthol hydrochloride	25	25
Aniline	5,000	500
Anthranilic acid	5,000	1,000
Cresidine salt	5,000	1,000
Pyrazolone T	5,000	1,000
R-amino salt	5,000	1,000
R-salt	5,000	1,000
Schaeffer's salt	5,000	1,000
Sodium naphthionate	5,000	500
Sulfanilamide	5,000	500
Sulfanilic acid	1,000	500
Miscellaneous dyes		
Methyl green	100	50
Methyl violet 2B	10	10
Nigrosin	5,000	500

TABLE 2. *Positive control chemicals for the tester strains with and without aroclor-treated rat S9*

Test compound	Amt per plate	No. of revertants per plate					
		TA1538		TA98		TA100	
		−S9	+S9	−S9	+S9	−S9	+S9
None		9	24	11	35	100	87
Dimethyl sulfoxide	0.1 ml	11	19	21	27	124	99
Sodium azide	0.5 μg	13	20	11	23	1,165	96
2-Nitrofluorene	5 μg	728	239	578	171	1,586	525
2-Aminoanthracene	2.5 μg	15	882	22	799	90	2,593

TABLE 3. *Historical mean values ± standard deviations for normal background reversions to prototrophy derived from dose-response data*

Strain	n	No. of revertant colonies					
		Cells only		Dimethyl sulfoxide		Twice mean values	
		−S9	+S9	−S9	+S9	−S9	+S9
TA1538	8	13 ± 4	24 ± 4	13 ± 3	22 ± 8	26	46
TA98	28	21 ± 5	38 ± 7	17 ± 5	38 ± 6	39	76
TA100	4	148 ± 13	156 ± 17	136 ± 7	150 ± 11	284	306

reversion to prototrophy of the three tester strains used throughout these studies.

Table 4 gives the mean values of representative dose-response curves. The dyes mutagenic with and without metabolic activation were lithol red, methyl yellow, methylene blue (USP), neutral red, and phenol red. Lithol red, methyl yellow, and phenol red were of moderate and about equal potency, methylene blue was somewhat more potent, and neutral red was an extremely potent mutagen when the liver enzymes were added. Orange II was of extremely low potency, and the addition of S9 inactivated the compound. Of the derivatives only 3-aminopyrazolone was a direct-acting mutagen.

Methyl red and 4-phenyl-azo-1-naphthylamine required activation, as did the derivatives 4-aminopyrazolone, 2,4-dimethylaniline, and *N,N*-dimethyl-*p*-phenylenediamine. The most potent were *N,N*-dimethyl-*p*-phenylenediamine, 2,4-dimethylaniline, and methyl red followed by 4-aminopyrazolone and 4-phenyl-azo-1-naphthylamine.

We have already shown that methyl orange can be converted by intestinal microorganisms to the mutagenic metabolite *N,N*-dimethyl-*p*-phenylenediamine (6). This compound can also be derived from methyl red and methyl yellow through azo reductase. We therefore examined the azo reductase activity of S9 liver microsomes with methyl red, methyl yellow, and methyl orange. The results are shown in Table 5. With short periods of incubation, the S9 liver microsomes were active in reducing methyl red, but inactive with methyl orange and methyl yellow. The results suggest that the mutagenicity of methyl red shown in Table 3 was due to *N,N*-dimethyl-*p*-phenylenediamine produced through azo reduction. The specificity of S9 liver microsomes toward these compounds is not known.

Since azo reductase is also active in intestinal anaerobes (6), tests were conducted to determine whether a specific strain of these microorganisms could convert methyl yellow into the mutagen *N,N*-dimethyl-*p*-phenylenediamine. After incubation with *Fusobacterium* sp. 2 the reduced methyl yellow was shown to be mutagenic in strains TA1538 and TA98 with metabolic activation. Only the results from strain TA1538 are shown in Table 6. Thin-layer chromatographic analysis showed that *N,N*-dimethyl-*p*-phenylenediamine and aniline were the metabolites. Aniline was not mutagenic (Table 2) (4).

DISCUSSION

Neutral red has been shown to be a teratogen in chicken embryos (17), to produce hyperglycemia in dogs (15), to increase the blood glucose level in rats (8), and to be mutagenic in the Ames assay (9). Methyl red is structurally similar to methyl yellow (dimethylaminoazobenzene), which is a potent liver carcinogen (3, 13). In the plate incorporation assay, methyl red is a more potent mutagen with metabolic activation than is methyl yellow.

Aniline is a metabolite of methyl yellow and 4-phenyl-azo-1-naphthylamine; sulfanilic acid can be obtained from orange II, sunset yellow, or tartrazine; sodium naphthionate can be generated from amaranth; 1-amino-2-naphthol hy-

TABLE 4. *Mean numbers of revertants per plate*

Test compound[a]	Strain	Mean no. of revertants per plate at dose (μg): Controls: Cells only −S9	Cells only +S9	Dimethyl sulfoxide −S9	Dimethyl sulfoxide +S9	5 −S9	5 +S9	10 −S9	10 +S9	25 −S9	25 +S9	50 −S9	50 +S9	100 −S9	100 +S9	250 −S9	250 +S9	500 −S9	500 +S9	1,000 −S9	1,000 +S9	2,500 −S9	2,500 +S9	5,000 −S9	5,000 +S9
Azo dyes																									
Lithol red	TA1538	13	24	14	25	15	27	18	24	17	23	20	21	26	25	24	30	40[b]	48[c]	25[c]	59[b]	50[b]	83[b]	52[b]	105[b]
Methyl red	TA98	20	46	18	39	19	35	19	57	15	68	22	127[b]	20	173[b]	27	472[b]	19	1,008[b]	9	2,531[b]	ND[d]	ND	ND	ND
Methyl yellow	TA98	17	37	19	32	15	41	24	42	21	57	14	84[b]	21	90[b]	18	91[b]	23	109[b]	28	111[b]	34	111[b]	42[b]	121[b]
Orange II	TA1538	14	25	13	24	12	23	15	23	12	16	10	17	18	23	16	23	16	29	24	29	30	38	44[b]	41
4-PA-1-NA	TA98	12	37	17	45	ND	ND	23	45	25	49	12	54	T[e]	49	T	58	T	135[b]	T	193[b]	ND	ND	ND	ND
Metabolites or derivatives of azo dyes																									
4-AP	TA98	32	25	15	36	23	38	12	48	24	42	19	56	30	75[b]	21	115[b]	22	224[b]	26	501[b]	ND	ND	ND	ND
3-AP	TA98	20	35	20	45	495[b]	530[b]	740[b]	929[b]	1,161[b]	1,864[b]	1,400[b]	2,514[b]	950[b]	3,589[b]	T	1,871[b]	T	T	T	T	ND	ND	ND	ND
2,4-DMA	TA100	153	154	131	147	153	241	156	280	142	333[b]	127	554[b]	153	597[b]	160	841[b]	182	1,074[b]	187	1,233[b]	ND	ND	ND	ND
DMPD	TA98	23	35	21	31	22	218[b]	14	373[b]	21	1,037[b]	27	2,632[b]	23	2,211[b]	17	1,545[b]	17	2,675[b]	22	1,544[b]	ND	ND	ND	ND
Miscellaneous dyes																									
Methylene blue (USP)	TA98	18	40	20	39	52[b]	180[b]	73[b]	105[b]	62[b]	192[b]	197[b]	117[b]	154[b]	298[b]	148[b]	189[b]	94[b]	156[b]	T	67	ND	ND	ND	ND
Neutral red	TA98	22	33	15	38	21	1,117[b]	25	2,676[b]	52[b]	15,399[b]	78[b]	19,053[b]	98[b]	36,141[b]	175[b]	4,329[b]	240[b]	791[b]	10	236[b]	ND	ND	ND	ND
Phenol red	TA98	18	32	15	37	18	33	20	50	20	29	22	23	29	28	26	51	31	45[b]	53[b]	55[b]	86[b]	106[b]	84[b]	117[b]

[a] Abbreviations used: 4-PA-1-NA, 4-phenylazo-1-naphthylamine; 4-AP, 4-aminopyrazolone; 3-AP, 3-aminopyrene; 2,4-DMA, 2,4-dimethylaniline; DMPD, *N,N*-dimethyl-*p*-phenylenediamine.

[b] Number of revertants is significant (at least twice the background).

[c] Number of revertants is enhanced (not quite twice the background).

[d] ND, Not done.

[e] T, Toxic.

TABLE 5. *Specific activity of azoreductase of liver S9*[a]

Substrate	Sp act (μmol/mg of protein/min)
Methyl red	0.94
Methyl yellow	0.06
Methyl orange	0.03

[a] Rat was pretreated with Aroclor 1254.

TABLE 6. *Mutagenicity of methyl yellow and a culture filtrate from an anaerobically grown culture tested with strain TA1538*

Test conditions	No. of revertant colonies	
	−S9	+S9
Cells only	11	18
Phosphate buffer	10	25
Methyl yellow[a] + phosphate buffer	6	18
Culture filtrate (methyl yellow[b] + phosphate buffer + *Fusobacterium* sp. 2[c])	14	1,494

[a] Methyl yellow was added at 0.25 μmol per plate test.

[b] Complete reduction after 1 h of incubation at 37°C with slow shaking.

[c] Dry weight of cells was 17.6 mg/ml.

drochloride is a metabolite of lithol red or orange II; *N,N*-dimethyl-*p*-phenylenediamine can be obtained from methyl red or methyl yellow; R-amino salt (1-amino-2-naphthol-3,6-disulfonic acid disodium salt) is a metabolite of amaranth; anthranilic acid is a metabolite of methyl red; Schaeffer's salt (2-naphthol-6-sulfonic acid, sodium salt) is an intermediate in the synthesis of sunset yellow; cresidine salt is an intermediate in the synthesis of allura red. Pyrazolone T, 4-aminopyrazolone, and 3-aminopyrene are structurally similar to a metabolite of tartrazine, *p*-sulfo-phenyl-3-carboxy-4-aminopyrazolone. *p*-Sulfo-phenyl-3-carboxy-4-aminopyrazolone is not stable (20), and it was not tested for mutagenicity. It is unlikely that 4-aminopyrazolone or 3-aminopyrene is produced from tartrazine (Fig. 1).

The commercially available compounds were of fairly high purity (±96%), but the possibility exists that impurities may be present in the concentrations above 1,000 μg per plate and may contribute to the mutagenicity of orange II with activation and methyl yellow without activation.

Hartman et al. showed that purified 2,4-dimethylaniline is mutagenic in the *Salmonella*-microsome test only at concentrations greater than 250 μg (10). In the present study, 2,4-dimethylaniline was mutagenic at 25 μg; impurities may be factors which contributed to the mutagenicity of this compound.

2-Amino-1-naphthalenesulfonic acid, the metabolite of lithol red, was not commercially available and was not tested for mutagenicity. However, 4-amino-1-naphthalenesulfonic acid (sodium salt) is structurally similar to 2-amino-1-naphthalenesulfonic acid and was not mutagenic in the present study.

Recent studies by McCoy et al. (18) have shown that anaerobic bacteria (*Clostridium perfringens* and *Bacteroides fragilis*) can activate a procarcinogen to a mutagen. Our data clearly indicate that *Fusobacterium* sp. 2 can convert methyl yellow into a product that can be metabolically activated to a mutagen. Methyl red, methyl orange, and methyl yellow are structurally similar, and all can be converted to the mutagenic metabolite *N,N*-dimethyl-*p*-phenylenediamine. Methyl red can be reduced by S9 azo reductase, whereas methyl red and methyl yellow cannot. When methyl yellow and methyl orange are incubated with intestinal anaerobes, both are converted to a mutagenic *N,N*-dimethyl-*p*-phenylenediamine (5).

ACKNOWLEDGMENTS

We are indebted to Mukund Shah for allowing us to use the data on methylene blue (USP), which we tested for him, with the data presented here. We also thank the late Carl Valentine as well as Corinthia Brown, James Fornwald, Robert Wolfe, Robert Stauffer, Kenneth Moats, Carol Smith, and Carol Roetzel for their valuable technical assistance and also Nancy Forletta for typing the manuscript.

This work was supported by Public Health Service grant N01-CO-75380 from the National Cancer Institute.

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