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Mutagenic Activities of Commercial Sudan III and Scarlet Red are Due to Impurities

MAMORU MIYAGOSHI, YUMIKO HAYAKAWA, MINORU NAGATA

and TOMIO NAGAYAMA

*Tokyo College of Pharmacy, 1432-1, Horinouchi,
Hachioji, Tokyo 192-03, Japan*

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Commercial samples and pure chemical samples of Sudan III (C.I. 26100) and Scarlet Red (C.I. 26105) were tested for mutagenic activities in the Ames test. Three out of five commercial samples of Sudan III were mutagenic to *Salmonella typhimurium* TA98 and TA100 in the presence of S9 mix, and all of the commercial samples of Scarlet Red tested were mutagenic to the two strains with microsomal activation. Neither of the purified dyes, however, was mutagenic to these strains with or without S9 mix. These results demonstrate that contaminants present in these commercial diazo-dyes were responsible for the mutagenic activity. Some of the contaminants were separated by preparative thin layer chromatography, and the starting materials of the azo-dyes, *i.e.* *p*-aminoazobenzene (AB) and *o*-aminoazotoluene (oAT), were found to be the main mutagenic contaminants.

Keywords—mutagenicity; Ames test; commercial azo-dyes; *p*-aminoazobenzene; *o*-aminoazotoluene; impurity; Sudan III; Scarlet Red

Introduction

Intensive studies have been carried out on the mutagenicities of azo-dyes by means of the Ames test.¹⁾ However, only a few papers²⁾ have referred to the mutagenic activity of the impurities contained in commercial dyes, such as starting materials, reaction intermediates and by-products. In our previous study,³⁾ commercial samples and purified chemical samples of Lithol Rubine B (C.I. 15850), Lithol Rubine BCA (C.I. 15850 : 1), Brilliant Lake Red R (C.I. 15800) and Deep Maroon (C.I. 15880) were tested for their mutagenic activities in the Ames test. The results suggested that the mutagenic activities of the commercial samples of these dyes were due to impurities contained in the dye samples.

In the present investigation, Sudan III (C.I. 26100) and Scarlet Red (C.I. 26105) were tested for mutagenicity according to the me-

thod reported previously.³⁾ Sudan III (1-(*p*-phenylazophenylazo)-2-naphthol; D & C Red No. 17; C.I. Solvent Red 23) has been used in coloring oils, spirit lacquers and also as a stain for zoological, pathological and vegetable materials such as wax, cutin and resin. Scarlet Red (1-(4-*o*-tolylazo-*o*-tolylazo)-2-naphthol; C.I. Solvent Red 24) has been used as a stain for fat. Use of these dyes as food additives is prohibited in Japan.⁴⁾ Sudan III and Scarlet Red are normally synthesized from *p*-aminoazobenzene (AB) and 2-naphthol,⁵⁾ and from *o*-aminoazotoluene (oAT) and 2-naphthol,⁶⁾ respectively, by diazo-coupling. 2-Naphthol has no mutagenic activity⁷⁾ but AB and oAT are known to be mutagenic.⁸⁾ Therefore the mutagenic activity of those two dyes might be due to residual starting materials (AB, oAT). In this work, commercial dye samples were subjected to HPLC and preparative TLC and the

Fig. 1. Chemical Structures of Diazo-Dyes and Their Starting Materials

a) Color index number.

separated impurities were tested for mutagenicity in the Ames test.

Materials and Methods

Pure Azo-Dyes—Sudan III and Scarlet Red (See Fig. 1 for chemical structure) were synthesized by diazo-coupling reaction,^{5,6)} then recrystallized three times from ethanol-benzene (5 : 2, v/v) and from ethanol, respectively.

Commercial Dye Samples—Commercial samples of Sudan III and Scarlet Red were kindly provided by Teijin Papilio Co., Ltd., Tokyo, Japan.

Characterization of Commercial Dye Samples—Determination of melting point (mp) and maximum absorption wavelength ($\lambda_{\max}$) in ethanol and elemental analysis were carried out in a usual manner. The pure dye content was determined colorimetrically and by HPLC. For spectrophotometric determination, ethanolic dye solutions (10 mg of purified or commercial dye in 10 ml of ethanol) were used. For HPLC, 10 μ l of a 100 ppm ethanolic dye solution was injected. The analytical conditions for HPLC were described in a previous report.⁹⁾ The purity of the dye samples was calculated by comparison of the peak height with that of the purified dye. The physicochemical characteristics of purified and commercial dyes are shown in Table I. The melting point of commercial dyes was normally lower than that of purified dyes. On the other hand, purified and commercial samples gave similar

UV absorption spectra and elemental analysis results.

Chemicals—Dimethylsulfoxide (DMSO) of spectrophotometric grade from Dojin Laboratories, Kumamoto, Japan, bacto agar and nutrient broth from Difco Laboratories, Detroit, Michigan, U.S.A., D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase and nicotin amide-adenine dinucleotide phosphate (reduced form) from Oriental Yeast Co., Ltd., Tokyo, Japan were used. All other reagents used were of extra-pure reagent grade.

Mutagenesis Assay—The assay was carried out according to the modified Ames test procedure.¹⁰⁾ *Salmonella typhimurium* tester strains were kindly provided by prof. Isao Tomita, Shizuoka College of Pharmacy, Shizuoka, Japan. The cells were grown in nutrient broth with shaking for 16 h at 37°C. A mixture of a 0.1 ml aliquot of the cell suspension, 0.1 ml of a test sample solution in DMSO and 0.5 ml of either 100 mM phosphate buffer (pH 7.4) or S9 mixture was gently mixed with 2 ml of soft agar, and the resulting mixture was poured onto a minimal agar plate. When the mixture contained S9, the mixture was preincubated at 37°C for 20 min before being mixed with soft agar and poured onto a minimal agar plate. S9 was prepared from the liver of male rats of SD strain treated with PCB.¹¹⁾ A control plate contained 0.1 ml of DMSO instead of a test solution. Colonies of His⁺ revertants were

TABLE I. Physicochemical Properties of Purified and Commercial Diazo-Dyes Used in This Study

Compound	mp (°C)	$\lambda_{\max}$ (nm) ^{a)}	Elemental analysis (%)			Pure dye content (%)	
			C	H	N	Method A	Method B ^{b)}
Sudan III							
—Pure ^{c)}	205	510	75.44	4.70	16.04	100	100
—1 ^{d)}	200	510	75.23	4.60	15.77	99.8	98.9
—2	200	510	74.08	4.86	14.83	93.4	94.5
—3	198	510	74.70	4.64	15.68	98.2	97.5
—4	203	510	74.85	4.52	15.85	100.3	99.4
—5	195	510	74.67	4.51	15.30	93.7	89.0
	(206.5—27.5) ^{e)}		(74.98	4.58	15.90) ^{f)}		
Scarlet Red							
—Pure ^{c)}	189	522	75.84	5.24	14.86	100	100
—1 ^{d)}	175	522	75.64	5.26	14.75	88.4	83.9
—2	173	522	75.66	5.35	14.33	86.8	81.5
—3	178	522	75.30	5.27	14.52	88.6	84.3
	(189) ^{e)}		(75.77	5.30	14.73) ^{f)}		

a) The maximal absorption wavelength in EtOH.

b) Method A: spectrophotometric determination at $\lambda_{\max}$. Method B: determination by HPLC.

c) Purified dye.

d) The numbers indicate the reference numbers of commercial dye samples.

e) Data from ref. 13.

f) Theoretical value.

counted after incubation at 37°C for 2 d. 4-Nitroquinoline-1-oxide (4NQO) and benzo[*a*]pyrene (B[*a*]P) were used as controls in each experiment to monitor the sensitivity of TA98 and TA100.

Thin Layer Chromatography—Samples were applied to Silica-gel 60F₂₅₄ TLC plates (20×20 cm, 0.25 mm thickness, purchased from Merck Co., Ltd.) and the plates were developed with trichloroethylene-methylene chloride-chloroform (4:2:1, v/v) for Sudan III and with carbon tetrachloride-chloroform-ethyl ether (3:2:1, v/v) for Scarlet Red at room temperature. The spots were detected under ultraviolet irradiation.

High-Performance Liquid Chromatography—HPLC separation was performed on a Nucleosil 5C₁₈ analytical HPLC column (15 cm × 4 mm i.d.) with a mobile phase of 6 parts of acetonitrile-THF (7:3, v/v) and 4 parts of water. The pH adjustment was done with phosphoric acid (pH 3.20). The column was operated at ambient temperature and the flow rate was 1.0 ml/min. The wavelength used for detection was 350 nm. Commercial dyes and the starting materials AB and oAT were each dissolved in ethanol (1000 ppm) and a 50 μ l aliquot

of sample solution was injected into the HPLC apparatus. The contents of AB and oAT were estimated by comparison of the areas of the peaks, calculated by means of a chromatogram processor (model 7000AS, System Instrument Co., Ltd., Tokyo Japan), with those of pure AB or oAT solutions of known concentration.

Separation and Identification of Contaminants in Commercial Dyes—Impurities in the commercial dyes were isolated by preparative thin layer chromatography (Silica-gel 60F₂₅₄ from Merck Co., Ltd., 20×20 cm, 2 mm thickness).

For Sudan III, the developing solvent system used was the same as that described above. For Scarlet Red, separation by TLC was carried out in two steps, with different solvent systems. First, the plate was developed with trichloroethylene-carbon tetrachloride-chloroform-dichloromethane (3:3:1:1, v/v), then the separated spots were scraped off the plate and extracted with chloroform. The chloroform extract was applied to a second TLC plate and the plate was developed with carbon tetrachloride-chloroform-ethyl ether (3:2:1, v/v). Each separated spot was scraped off the second

Fig. 2. TLC Patterns of Diazo-Dyes and Their Starting Materials

Trichloroethylene-methylene chloride-chloroform (4:2:1, v/v) was used as a solvent system for Sudan III and carbon tetrachloride-chloroform-ethyl ether (3:2:1, v/v) for Scarlet Red.

The shadowed spots are those of the pure dyes.

TLC plate and treated with chloroform or methanol until the adsorbed dye was completely extracted from the silica-gel. Each extract was evaporated under a vacuum to yield the individual dye contaminants in fairly good purity.

Results and Discussion

Purity of Commercial Dyes

In Japan, Sudan III and Scarlet Red of better than 85% purity are officially approved for medical and cosmetic uses.⁴⁾ HPLC and colorimetric measurement showed that the purity of all the commercial Sudan III dyes tested was well above 85%, while that of Scarlet Red was below the official limit.

Fig. 3. HPLC Chromatograms of Commercial Sample No. 5 of Sudan III and No. 1 of Scarlet Red

Operating conditions—column, Nucleosil 5C₁₈ (15 cm × 4 mm i.d.); mobile phase, 70% acetonitrile-THF (7:3, v/v), 30% water (pH 3.20, phosphoric acid); flow rate, 1.0 ml/min; detection wavelength, 350 nm; column temperature, ambient.

TABLE II. Content of Starting Materials in Commercial Azo-Dyes

Compound	Content of starting materials	
	AB (%)	oAT (%)
Sudan III		
-1 ^{a)}	0.016	-
-2	0.023	-
-3	0.070	-
-4	0.066	-
-5	0.121	-
Scarlet Red		
-1	-	0.088
-2	-	0.020
-3	-	0.015

^{a)} The numbers indicate the reference numbers of commercial dye samples.

Thin Layer Chromatography of Commercial Dyes

Thin layer chromatograms of 5 commercial Sudan III and three commercial Scarlet Red samples are shown in Fig. 2.

Both commercial Sudan III and Scarlet Red contained at least seven colored substances. The spots on the plate of Sudan III

were designated as 5-1—5-7 from top to bottom. The spots 5-5 and 5-6 were identified as that of pure dye and that of AB, respectively. A commercial sample of Scarlet Red gave three spots (orange, red and brown spots) on

the first step thin layer plate (Fig. 2B). When each spot was subjected to the second thin layer chromatography, the orange, red and brown spots gave six (O-1...O-6), four (B-1...B-4) and six (R-1...R-6) spots respectively (Fig.

TABLE III. Mutagenic Activities of Diazo-Dyes and Their Starting Materials

Test compound	Concentration ($\mu\text{g}/\text{plate}$)	Number of His ⁺ revertants/plate			
		TA98		TA100	
		-S9 ^{a)}	+S9 ^{b)}	-S9	+S9
DMSO ^{c)}	-	24 $\pm$ 7 (32) ^{d)}	41 $\pm$ 10 (23)	107 $\pm$ 25 (30)	106 $\pm$ 25 (25)
4NQO ^{e)}	0.05	-	-	409 $\pm$ 102 (29)	-
	0.10	186 $\pm$ 21 (30)	-	600 $\pm$ 123 (30)	-
	0.20	241 $\pm$ 40 (28)	-	-	-
B[a]P ^{e)}	2	-	237 $\pm$ 95 (24)	-	419 $\pm$ 179 (27)
	5	-	931 $\pm$ 247 (28)	-	844 $\pm$ 192 (26)
Sudan III -Pure ^{f)}	5	27 ^{g)}	51	100	100
	50	24	54	99	107
	500	21	46	96	105
	1000	21	42	104	110
	5000	20	49	109	100
Sudan III -1 ^{h)}	5	19	27	99	99
	50	23	39	109	101
	500	21	53	103	136
	1000	25	42	121	101
	5000	34	34	115	111
Sudan III -2	5	22	38	100	103
	50	25	50	97	113
	500	20	49	99	113
	1000	22	52	106	137
	5000	28	34	111	102
Sudan III -3	5	23	39	92	104
	50	29	40	102	112
	500	39	51	98	132
	1000	49	72 ⁱ⁾	98	218
	5000	46	106	126	237
Sudan III -4	5	24	38	92	104
	50	20	46	101	126
	500	21	57	111	170
	1000	23	73	125	215
	5000	27	111	117	246
Sudan III -5	5	26	40	118	126
	50	24	64	114	149
	500	41	73	112	205
	1000	58	99	112	239
	5000	46	202	126	278

TABLE III. (continued)

Test compound	Concentration ($\mu\text{g}/\text{plate}$)	Number of His ⁺ revertants/plate			
		TA98		TA100	
		-S9	+S9	-S9	+S9
Scarlet Red -Pure	5	30	43	108	109
	50	29	46	105	107
	500	25	51	110	102
	1000	27	41	108	103
	5000	22	46	100	100
Scarlet Red -1	5	29	46	98	94
	50	22	57	84	136
	500	22	<u>139</u>	84	<u>216</u>
	1000	21	<u>177</u>	97	<u>282</u>
	5000	24	<u>198</u>	106	<u>300</u>
Scarlet Red -2	5	28	50	104	111
	50	35	56	115	104
	500	32	<u>94</u>	115	<u>104</u>
	1000	41	<u>97</u>	126	<u>208</u>
	5000	38	<u>113</u>	115	<u>219</u>
Scarlet Red -3	5	21	44	115	96
	50	23	41	120	92
	500	33	<u>85</u>	137	141
	1000	24	<u>95</u>	101	<u>220</u>
	5000	43	<u>144</u>	98	<u>216</u>
AB	1	27	44	119	106
	10	29	<u>112</u>	88	<u>207</u>
	100	29	<u>256</u>	82	<u>439</u>
oAT	1	22	45	95	112
	10	25	<u>385</u>	85	<u>465</u>
	100	killing	<u>1531</u>	killing	<u>1859</u>

a) -S9: without S9 mix.

b) +S9: with S9 mix.

c) DMSO: solvent control.

d) Mean $\pm$ S.D. (number of experiments).

e) 4NQO: reference compound without S9 mix. B[a]P: reference compound with S9 mix.

f) Purified dye.

g) The numbers of revertant colonies are the averages of two assays.

h) The numbers indicate the reference numbers of commercial dye samples.

i) Data underlined indicate that the dye was mutagenic at the concentration tested.

2C). The spot R-2 corresponded to pure Scarlet Red and R-3 to oAT.

HPLC of Commercial Dyes

HPLC (Fig. 3) confirmed the presence of AB or oAT in commercial Sudan III or in commercial Scarlet Red samples, respectively. A number of peaks were observed in addition to the main peaks in these chromatograms, and presumably represent the starting mate-

rials, reaction intermediates or by-products. The amount of total contaminants in commercial dyes estimated by HPLC ranged from 0.6% to 18.5% (Table I). The content of AB or oAT was in the range of 0.015%–0.121% based on the total dye specimens (Table II).

Mutagenicity Assay

The results of Mutagenicity assay of purified and commercial dyes, and their starting

TABLE IV. Mutagenic Activities of Impurities in Commercial Dyes Separated by Preparative TLC

Test compound	Concentration ($\mu\text{g}/\text{plate}$)	Number of His ⁺ revertants/plate	
		TA98 (+S9) ^{a)}	TA100 (+S9)
5-6	1	44 ^{b)}	125
	10	73 ^{c)}	184
	100	<u>188</u>	<u>243</u>
R-3	1	61	96
	10	<u>117</u>	158
	100	<u>250</u>	<u>551</u>
R-4	1	59	116
	10	<u>178</u>	109
	100	<u>3320</u>	<u>577</u>
B-3	1	59	128
	10	<u>81</u>	124
	100	<u>201</u>	195
M-R-4	0.1	42	110
	1	<u>85</u>	108
	10	<u>810</u>	<u>215</u>

a) +S9; with S9 mix.

b) The numbers of revertant colonies are the averages of two plates.

c) Data underlined indicate that the dye was mutagenic at the concentration tested.

materials are summarized in Table III.

Samples No. 3, 4 and 5 of commercial Sudan III and all of the commercial Scarlet Red samples tested were mutagenic to TA98 and TA100 with S9 mix. Since neither of the purified dyes was mutagenic, the mutagenicity of the commercial dyes can be ascribed to the impurities contained in them. AB and oAT are known to be mutagenic in the presence of S9 mix, as reported by Yahagi *et al.*⁸⁾

In order to investigate whether or not any contaminants in commercial Sudan III and Scarlet Red other than the starting materials possess mutagenicity, the chromatographically separated substances were further tested for mutagenic activity.

Of the seven spots (5-1...5-7) obtained on a thin layer plate of commercial Sudan III

Fig. 4. HPLC Chromatograms of the Substances Separated by preparative TLC from Commercial Sample No. 1 of Scarlet Red

Operating conditions—column, Nucleosil 5C₁₈ (15 cm × 4 mm i.d.); mobile phase, 60% acetonitrile-THF (7:3, v/v), 40% water (pH 3.20, phosphoric acid); flow rate, 1.0 ml/min; detection wavelength, 350 nm; column temperature, ambient.

(Fig. 2), spot 5-6 was identified as AB and was mutagenic to both tester strains with S9 mix (Table IV). Other unidentified substances *i.e.* 5-1...5-4 and 5-7 did not show clear mutagenic activities, though they appeared to slightly increase the number of his⁺ revertants. It can be concluded that the mutagenicity of commercial samples of Sudan III is mainly due to AB contained in the dye.

Samples No. 1 and No. 2 of commercial Sudan III showed no mutagenic activity, probably because their contents of AB (0.016% and 0.023%) were quite small. The minimum concentration at which AB shows mutagenic

activity is 10 $\mu\text{g}/\text{plate}$. However, 500 $\mu\text{g}/\text{plate}$ of No. 5 Sudan III sample, which would contain only 0.6 μg of AB, showed mutagenic activity. This finding suggests that some other unidentified mutagenic substance is present in these dye samples.

Of a number of spots obtained from commercial Scarlet Red dyes on TLC, R-3 was mutagenic to both tester strains with S9 mix (Table IV). R-4 and B-3 were mutagenic after activation with S9 mix (Table IV). The mutagenic activity of R-4 towards TA98 with S9 mix was stronger than that of R-3, but the activity of R-4 towards TA100 with S9 mix was weaker than that of R-3. HPLC showed that the mutagenic fractions R-3, R-4 and B-3 were not pure compounds, but mixtures (Fig. 4).

The main substance in R-4 (M-R-4) was also found to be contained in R-3 and B-3. The peak height of M-R-4 was about the same as the peak height of oAT in R-3, but was quite small in B-3. A small amount of

oAT was found in R-4. M-R-4 obtained by HPLC showed mutagenic activity towards the two strains with S9 mix. M-R-4 is thus a strongly active unidentified compound. Therefore it is suggested that the mutagenic activity of B-3 is due to M-R-4 contaminating B-3 and that the activity found in R-3 is due to oAT and M-R-4. It may be concluded that the mutagenic activity of commercial Scarlet Red is due to contamination by oAT (the starting material) and an unidentified contaminant M-R-4. Some other contaminants may also possess mutagenic activity. However, the quantities obtained were too small for mutagenicity assay to be performed. It is known that oAT is a potent carcinogen,¹²⁾ and contamination by the highly mutagenic compound M-R-4 and oAT in commercial Scarlet Red samples was demonstrated in this paper. Accordingly, care should be taken when commercial Scarlet Red is used in medical or cosmetic preparations.

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