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Evaluation of New Metallized Direct Dyes for Mutagenicity Using the Salmonella Mammalian Mutagenicity Assay

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Abstract: A series of new metallized direct dyes based on benzidine congeners, 2,2'-dimethyl-5,5'-dipropoxybenzidine and 5,5'-dipropoxybenzidine, were evaluated for mutagenicity in Salmonella typhimurium strains TA98 and TA100. All of the dyes examined were judged to be non-mutagenic with and without metabolic activation while toxicity was seen in some dyes at high doses. The study also suggested that the standard Salmonella mutagenicity plate-incorporated assay was an excellent method for evaluation of dyes for mutagenicity.

Keywords: Direct dyes, Copper complex dyes, Mutagenicity, Salmonella, Benzidine, Genotoxicity

Introduction

Direct dyes have been used to dye cellulose for over 100 years. Owing to the ease of their application and the wide gamut of colors available at a modest cost, direct dyes are still a popular dye class [1]. Most direct dyes have disazo and trisazo structures, with each color dominated by unmetallized structures [2]. For many years, direct dyes included those made from benzidine and its analogs. The resultant dyes were used for textiles, paper, leather and plastics [3,4]. Nowadays, it is well known that benzidine is both a mutagenic amine and a human carcinogen [5-13].

Although benzidine has not been manufactured for sale in the United States since the mid-1970s, benzidine-based dyes are still used in many countries. In fact, it has been reported that more than 90 azo dyes based on benzidine and benzidine congeners are used in the US [14]. Benzidine, a synthetic chemical that does not occur naturally, is carcinogenic to a variety of mammalian species including humans [11,13,14]. Three compounds closely related to benzidine, 3,3'-dimethylbenzidine (*ortho*-tolidine), 3,3'-dimethoxybenzidine (*ortho*-dianisidine) and 3,3'-dichlorobenzidine (cf. Figure 1) also are used in the manufacture of commercial dyes and pigments. There is evidence that these three chemicals are carcinogenic in laboratory animals, although evidence of their ability to induce cancer in humans is not available [13].

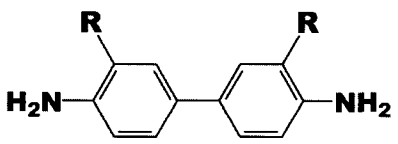

Figure 1. Structures of key benzidine homologs; R=H (benzidine), CH_3 (*ortho*-tolidine), OCH_3 (*ortho*-dianisidine).

The commercial utility of benzidine-based colorants and concern over their potential health risks have caused the search for viable nonmutagenic analogs of benzidines to be an important research problem [15-21]. In this regard, it has been shown that 2,2'-dimethyl-5,5'-dipropoxybenzidine (cf. Figure 2) is non-mutagenic [22] and gives non-mutagenic azo and azomethine pigments [23]. In previous studies in our laboratories, a series of new direct dyes including copper complex dyes was synthesized from non-genotoxic diamines and evaluated as technical alternatives to certain benzidine-based dyes [24,25].

Of the numerous short-term genotoxicity assays, a microbial mutagenicity assay developed by Bruce Ames and co-workers has become the most widely used and most thoroughly investigated [26-28]. The Salmonella/mammalian microsome mutagenicity assay uses specially engineered strains of the bacterium Salmonella typhimurium to screen both individual compounds and mixtures for mutagenic and carcinogenic potentials. The assay which is rapid, inexpensive, and reliable possesses a vast database. The Environmental Mutagen Information Center (EMIC) database lists publications with test data on more than 6000 pure chemical compounds. In addition, Salmonella assay information for numerous complex environmental mixtures has been published [29-36].

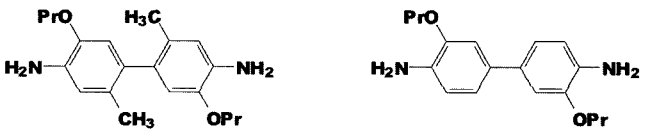

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Figure 2. Structures of 2,2'-dimethyl-5,5'-dipropoxybenzidine (**1**) and 5,5'-dipropoxybenzidine (**2**).

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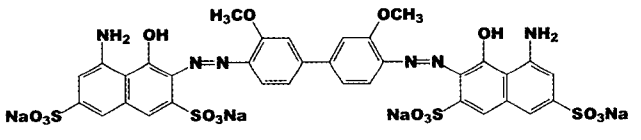
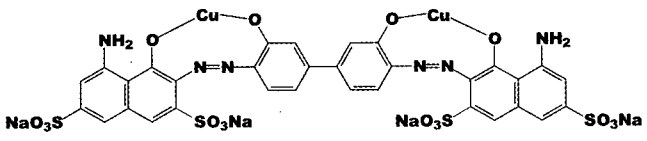
In the standard approach to Salmonella mutagenicity testing, a culture of a bacterial tester strain is mixed with the test agent and an optional exogenous metabolic activation system (e.g. S9) in a molten agar overlay. The overlay contents are then poured onto minimal-histidine agar plates and, following incubation, the plates are scored for mutant colonies (revertants). By testing incremental doses of the test agent on a series of plates, dose-response relationships may be examined. Several modifications of the standard assay protocol have been reported [37-45].

In the past paper from our laboratories [46,47], the effect of bulky alkoxy substituents on the mutagenicity of a series of 4-aminoazobenzene (**AAB**) derivatives and 1,4-phenylenediamine (**PD**) derivatives was investigated. This study showed that the replacement of bulky alkoxy group in the 3-position of **AAB** derivatives and in the 2-position of the **PD** derivatives leads to a significant decrease in mutagenic activity. Shahin and co-workers have obtained similar results for a series of *meta*-phenylenediamine derivatives [15,16]. The present study extends this approach to decreasing mutagenicity to a series of new direct dyes. This work employs the Salmonella typhimurium mutagenicity assay with strains TA98 and TA100. The new dyes tested contain 2,2'-dimethyl-5,5'-dipropoxybenzidine (**1**) and 5,5'-dipropoxybenzidine (**2**) as potential alternatives to benzidine and several coupling agents such as J-acid, Chromotropic acid, H-acid, Neville-Winther acid, Naphthionic acid, and Gamma acid. The summary of mutagenicity test result including the average number of mutant colonies (revertants) at each doses are provided for all the dyes.

Results from other studies [9] indicate that the formation of copper complexes of certain *ortho*-dianisidine-based dyes can influence genotoxicity. For example, while C.I. Direct Blue 15 is mutagenic, its bis-copper complex C.I. Direct Blue 218 is not (cf. Figure 3). With this in mind, copper-complexed forms of these new direct dyes were tested for mutagenicity with and without exogenous metabolic activation (cf. Figure 4) [25].



C.I. Direct Blue 15 (mutagenic)



C.I. Direct Blue 218 (non-mutagenic)

Figure 3. Example of the effect of copper-complex formation on direct dye mutagenicity.

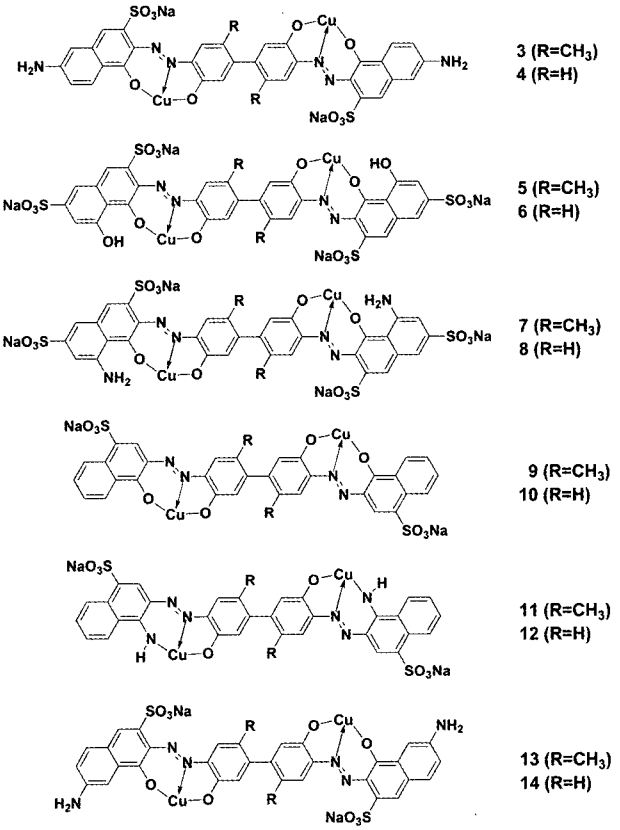


Figure 4. Structures of Cu complexed direct dyes derived from diamines **1** and **2**.

Materials and Methods

Chemicals

All dyes tested are novel and were synthesized in our laboratory. Figure 4 shows the structure of all 12 metallized direct dyes (**3-14**) tested. The structure of each dye was confirmed by atomic absorption and neutron activation analysis, the details of which are shown in other publications [24,25]. The purity of the novel dyes was confirmed by thin-layer chromatography (TLC).

Mutagenicity Methods

The Salmonella mutagenicity plate-incorporated assay with and without exogenous activation was performed using the procedures of Maron and Ames [28]. All the dyes were tested at least twice, on separate days, in Salmonella typhimurium strains TA98 and TA100. The Salmonella typhimurium strains were kindly supplied by Dr. B. N. Ames of the Department of Biochemistry, University of California, DA. The activation system used was a 9000 g (S9) liver homogenate from Aroclor 1254-induced CD-1 male rats (MolTox Corporation, Boone, NC). The positive controls were sodium azide (for TA100, -S9), 2-nitrofluorene (for TA98, -S9), and 2-anthramine (for TA100 AND TA98, +S9). Chemicals were tested at levels up to at least

5 mg/plate at a minimum of 7 doses using triplicate plates at each dose level. Toxicity to (killing of) the bacteria was detected in 3 ways. At the time of plate counting, a visual thinning of the background lawn and/or the appearance of pin-point colonies that could not be demonstrated to be true revertants was registered as toxicity. Also, if the dose-response curve or the final segment of the dose-response curve produced a significant negative slope value, the associated doses were assumed to be toxic. Appropriate negative (solvent) and positive controls were run concurrently with each assay. All of the test compounds were dissolved in dimethyl sulfoxide (DMSO) just prior to use. Results were designated as positive, negative, or equivocal according to the criteria of Claxton and co-workers [48]. A test compound was not determined to be positive (mutagenic) or negative unless reproducible results were obtained in at least one strain/activation combination. To be designated as mutagenic, a dye must have produced an average revertant count that was more than two times the background average (i.e., the number of colonies at the 0-mg doses). In addition, a response was determined to be positive when there was a dose-related increase in revertant counts as determined by the statistical models of Bernstein and co-workers [49]. Under these guidelines [50], a response was considered equivocal when 1) test results were not reproducible, 2) there was a reproducible low-level increase in colony forming units but no dose-related response, or 3) when an increase was observed at only one dose. The method of Bernstein and co-workers [49] was used to assign a definitive slope value when the statistical models and visual examination of the data confirmed that the test compound was mutagenic.

Results and Discussions

Table 1-12 in the appendix provide a summary of the results obtained and slope values from the tests. The appendix contains the untransformed data for one of the definitive tests. At higher dose levels, coloration of the plates was intense. This meant that precipitates were somewhat difficult to distinguish from colored colonies and in some cases, an automatic colony counter could not be used for accurate counting.

Some of the copper complexed dyes precipitated within the agar overlay. For instance, the metallized dyes based on naphthionic acid (**11** and **12**) and gamma acid (**13** and **44**) precipitated at the 3-5 mg and 5 mg dose levels respectively. All other dyes did not precipitate until higher doses were reached. See the data in the Appendix for the precise doses at which the various dyes precipitated.

Toxicity

Toxicity was observed primarily when high doses (3-5 mg) of certain dyes were used in both strains with and without exogenous activation.

For the dyes, toxicity was observed mainly in the absence of exogenous metabolic activation. Dyes **3-5**, **7-8**, and **11-12** exhibited toxicity at 3-5 mg dose levels with strains TA98 and TA100 in the absence of exogenous metabolic activation. Also, dyes **8** and **11** exhibited toxicity at 3-5 mg in TA98 and TA100 in the presence of exogenous activation while dye **9** and dye **12** showed toxicity at 5 mg in strains TA98 and TA100, respectively, with S9 activation.

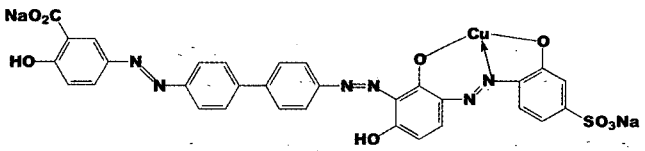


Figure 5. Structure of C.I. Direct Brown 95.

Mutagenicity

No positive or equivocal mutagenicity results were observed for the dyes tested in TA98 and TA100, in the presence or absence of exogenous metabolic activation.

The mutagenicity results of the dyes **3-14** in TA98 and TA100 with and without exogenous metabolic activation were consistent with results from testing C.I. Direct Blue 218. However, it should be noted that not all azo dyes will necessarily be rendered nonmutagenic solely by the formation of the corresponding copper complex. In this regard, the benzidine-based dye C.I. Direct Brown 95 is a copper complex, but the position of copper in the dye molecule is such that free benzidine can be released after azo reduction [9] (cf. Figure 5).

Conclusions

The results of this study indicate that the nonmutagenic benzidine analogs 2,2'-dimethyl-5,5'-dipropoxybenzidine and 5,5'-dipropoxybenzidine are potential replacements for benzidine, a potent mutagen, in the preparation of nonmutagenic azo dyes. New metallized direct dyes derived from the two analogs were shown to be nonmutagenic in the Salmonella mutagenicity assay in TA98 and TA100 in the presence or absence of S9. Although a thorough examination of the toxicological properties of these dyes is required before they can be deemed viable commercial products, clearly the present results provide an important step towards that end.

Acknowledgements

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Appendix

This section provides tables that give the mean, standard deviation, and slope values calculated using the method of Bernstein [49] to assess the mutagenicity for direct dyes **3-26** in Salmonella strains TA98 and TA100. A slope value is only reported if the test results are positive (mutagenic). Although replicate tests were performed as described in the text, only data from a single definitive test are represented in order to conserve space. Abbreviations used are as follows: **-S9**, no exogenous metabolic activation; **+S9**, exogenous metabolic activation; **STD**, standard deviation; **T**, toxicity; **P**, precipitate on plate; **NEG.**, negative result.

The positive controls employed were sodium azide (for TA100, -S9), 2-anthramine (for TA98 and TA100, +S9), and 2-nitrofluorene (for TA98, -S9). A negative solvent control of 100 μl dimethyl sulfoxide (DMSO) was also used.

Table 1. Mutagenicity test data for dye 3

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	32.6	4.9	34.3	1.5	74	6.2	81.6	6.6
0.1	34.5	0.7	35	2.8	94.5	19.1	95.5	4.9
0.3	36	11.3	33.5	6.4	97	14.1	99	2.8
0.5	22.5	7.8	33	1.4	93	2.8	117	2.8
1	16	2.8	26.5	10.6	92	2.8	106.5	7.8
3	8t	2.8	16.5	2.1	38.5t	12.1	52	2.8
5	6.5t	4.9	18.5	7.8	4.5t	3.5	59.5	10.6
Positive control	873.3	28.4	1553.3	38.8	851.7	62.1	1365	74.6
Slope	NEG.		NEG.		NEG.		NEG.	

Table 2. Mutagenicity test data for dye 4

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	32.6	4.9	34.3	1.5	74	6.2	81.6	6.6
0.1	30.5	0.7	38	14.1	96	18.3	106.5	2.1
0.3	21	9.8	31	9.9	123.5	9.1	109.5	12.1
0.5	29	2.8	22.5	0.7	114	8.5	113	4.2
1	26	5.6	22.5	6.3	96	4.2	109.5	24.7
3	20t	1.4	27	2.8	88	5.6	110.5	9.1
5	11.5t	4.9	18.5	3.5	48t	8.4	104	11.3
Positive control	873.3	28.4	1553.3	38.8	851.6	62.1	1365	74.6
Slope	NEG.		NEG.		NEG.		NEG.	

Table 3. Mutagenicity test data for dye 5

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	31.5	2.1	36.5	12.1	101	18.3	104.5	3.5
0.1	24	2.8	25.5	0.7	113	5.6	122	4.2
0.3	25	7.1	36.5	6.4	111.5	4.9	109	9.9
0.5	24	1.4	29.5	4.9	107	28.2	121.5	4.9
1	18	5.6	33.5	2.1	77	14.1	89	12.7
3	7t	1.4	20.5	0.7	41t	15.6	95	21.2
5	1.5t	0.7	20.5	0.7	2t	1.4	67	4.2
Positive control	815	49.5	907.5	24.7	915	7.1	1498.5	40.3
Slope	NEG.		NEG.		NEG.		NEG.	

Table 4. Mutagenicity test data for dye 6

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	31.5	2.1	36.5	12.1	101	18.3	104.5	3.5
0.1	24.5	7.8	42.5	3.5	112	4.2	103.5	3.5
0.3	25	2.8	33.5	2.1	109	1.4	109.5	10.6
0.5	27	4.2	31	8.4	107	5.7	110	2.8
1	30.5	10.6	34	8.5	119	1.4	120	1.4
3	25	9.9	32	1.4	93	8.5	127.5	4.9
5	23.5	7.8	30.5	6.3	88	5.7	114.5	6.4
Positive control	815	49.4	907.5	24.7	915	7.1	1498.5	40.3
Slope	NEG.		NEG.		NEG.		NEG.	

Table 5. Mutagenicity test data for dye 7

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	25	4.2	43	7.1	89.5	2.1	96	12.7
0.1	29	8.5	38.5	0.7	121	8.5	119.5	13.4
0.3	31	1.4	29.5	6.4	118	2.8	119	15.6
0.5	24	9.9	26.5	4.9	127	9.9	116	1.4
1	23	2.8	25.5	9.2	93	1.4	135.5	6.4
3	14.5t	2.1	22.5	2.1	27t	9.8	97	5.7
5	2.5t	0.7	7t	1.4	12t	14.1	75	21.1
Positive control	842.5	38.9	1385	21.2	874.5	41.7	1365	49.5
Slope	NEG.		NEG.		NEG.		NEG.	

Table 6. Mutagenicity test data for dye 8

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	25	4.2	43	7.1	89.5	2.1	96	12.7
0.1	24.5	0.7	32.5	3.5	111.5	2.1	115.5	6.3
0.3	33	1.4	30	2.8	109.5	6.4	127.5	0.7
0.5	27.5	0.7	30.5	3.5	107.5	14.8	114.5	6.3
1	19	4.2	24.5	3.5	97	18.4	107	2.8
3	1.5t	0.7	22t	2.8	5.5t	0.7	87	18.3
5	0.5t	0.7	8t	2.8	2t	0	46t	5.6
Positive control	842.5	38.8	1385	21.2	874.5	41.7	1365	49.5
Slope	NEG.		NEG.		NEG.		NEG.	

Table 7. Mutagenicity test data for dye 9

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	30	4.4	35.3	3.2	91.6	9.1	104	5.3
0.1	31	4.2	32	4.2	123.5	16.2	116.5	12.1
0.3	33.5	9.2	34.5	6.4	128.5	2.1	123.5	4.9
0.5	33.5	2.1	38.5	2.1	124.5	4.9	114	5.7
1	36.5	6.4	33.5	2.1	122.5	17.7	128.5	9.2
3	19.5	7.8	21.5	6.4	109.5	24.7	115.5	20.5
5	18	4.2	22	2.8	96.5	6.4	95.5	9.2
Positive control	715	48.2	1606.6	102.1	1126.7	35.1	1413.3	110.1
Slope	NEG.		NEG.		NEG.		NEG.	

Table 8. Mutagenicity test data for dye 10

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	28.3	5.5	38.3	3.8	104	6.1	104.6	11.7
0.1	29	7.1	34	2.8	97	1.4	127	7.1
0.3	30.5	2.1	37	4.2	106	2.8	128	4.2
0.5	32.5	7.8	42	2.8	105.5	3.5	129	1.4
1	39.5	9.2	37.5	0.7	98.5	19.1	154	5.7
3	28.5	2.1	32	11.3	100.5	13.4	145.5	6.4
5	31.5	3.5	37	4.2	95	14.1	155	16.4
Positive control	888.3	7.6	1756.7	119.3	773.3	25.1	1780	60
Slope	NEG.		NEG.		NEG.		NEG.	

Table 9. Mutagenicity test data for dye 11

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	29.5	0.7	32.5	0.7	98	4.2	102.5	6.4
0.1	27.5	3.5	29.5	4.9	104.5	4.9	115.5	7.8
0.3	28	4.2	23	4.2	89	7.1	121	1.4
0.5	28	8.5	24	4.2	97.5	7.8	97	2.8
1	20	5.6	20	7.1	82	16.9	125	21.2
3	13.5p,t	0.7	24p	1.4	37p,t	24.1	83p	2.8
5	13.5p,t	7.8	16p,t	2.8	19p,t	2.8	56p,t	9.9
Positive control	160.5	0.7	1235.5	33.2	695	7.1	1465	7.1
Slope	NEG.		NEG.		NEG.		NEG.	

Table 10. Mutagenicity test data for dye 12

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	29.5	0.7	32.5	0.7	98	4.2	102.5	6.4
0.1	37.5*	3.5	37	4.2	99.5	10.6	107	4.2
0.3	25.5	10.6	33	5.6	77.5	6.3	148.5	30.4
0.5	25.5	14.8	24	5.6	92	1.4	120.5	13.4
1	22	1.4	23	5.6	51.5	3.5	81.5	9.2
3	14p,t	5.6	16.5p	0.7	35.5p,t	6.3	39p,t	1.4
5	13.5p,t	0.7	22p	1.4	2p,t	3.5	25p,t	7.1
Positive control	160.5	0.7	1235.5	33.2	695	7.1	1465	7.1
Slope	NEG.		NEG.		NEG.		NEG.	

Table 11. Mutagenicity test data for dye 13

Dose (mg/plate)	TA 98				TA 100			
	-S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	28.3	5.5	38.3	3.8	104	6.1	104.6	11.7
0.1	27	2.8	34.5	12.1	130.5	7.8	124	32.5
0.3	26.5	2.1	38	4.2	131	22.6	160	28.3
0.5	34.5	6.4	34	1.4	104.5	0.7	141	12.7
1	35.5	4.9	35.5	2.1	110.5	2.1	174	15.6
3	35.5p	7.8	29p	1.4	144.5p	17.6	128.5p	2.1
5	30.5p	2.1	25.5p	7.8	125.5p	7.8	120.5p	3.5
Positive control	888.3	7.6	1756.7	119.3	773.3	25.2	1780	60
Slope	NEG.		NEG.		NEG.		NEG.	

Table 12. Mutagenicity test data for dye 14

Dose (mg/plate)	TA 98				TA 100			
	−S9		+S9		−S9		+S9	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
0	30	4.4	35.3	3.2	91.6	9.1	104	5.3
0.1	28.5	4.9	41.5	2.1	118	4.2	129	1.4
0.3	30	9.9	43.5	9.2	109	15.6	123	8.5
0.5	33	1.4	42.5	0.7	117.5	3.5	123.5	9.1
1	27.5	6.4	25.5	0.7	111.5	4.9	112	2.8
3	22.5	3.5	26	2.8	96.5	10.6	113	4.9
5	21.5p	0.7	17p	1.4	78p	8.5	97p	2.8
Positive control	715	48.2	1606.7	102.1	1126.7	35.1	1413.3	110.1
Slope	NEG.		NEG.		NEG.		NEG.	