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MUTAGENIC AND GENOTOXIC ACTIVITY DETECTED BY THE AMES, MICRONUCLEUS AND SCE TESTS UNDER THE INFLUENCE OF SAMPLES OF DYES MANUFACTURED IN POLAND

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Key words: Basic Blue 26, 44045, Acid Violet 49, 42640, Direct Red 83, 29225, Acid Black 194, Disperse Blue 37, Mutagenicity, *Salmonella typhimurium*, Genotoxicity, Mouse bone marrow

Abstract. In this study, we evaluated the mutagenic and genotoxic potential of commercial samples of chemicals manufactured by Polish dyestuff industry for their ability to mutate *Salmonella typhimurium* strains (TA97a, TA98, TA100 and TA102), and to induce formation of micronuclei (MN) and sister chromatid exchanges (SCE) in mice bone marrow cells. This study involved five dyes selected from a list of colorants, considered to be representative of those which were most extensively used in the trade. According to criteria listed by IARC (1984), the results obtained in this work and earlier tests of Basic Blue 26, 44045 and Direct Red 83, 29225 provided no evidence of genetic activity (all 3 tests were negative); for Acid Violet 49, 42640 and Disperse Blue 37, the evidence of genetic activity was inadequate (1 test was positive) and the tests for Acid Black 194 provided limited evidence of genetic activity (2 positive tests).

INTRODUCTION

At present there is no legally sanctioned obligation to assay genotoxic potential of chemicals manufactured and/or used in Poland. Genotoxicity assays are usually performed in research centres as part of their statutory activity, research or implementation work including, among other things, development of new research techniques. The assays are also ordered by commercial companies intending to sell their chemical products in countries where genotoxic assay certificates are obligatory. This work determines the genotoxic activity of commercial dyes manufactured in Poland by the test strategy and evaluation criteria recommended by the International Agency for Research on Cancer (8).

The mutagenic and genotoxic properties of tested dyes are confirmed in a combination of complementary assays using different biological endpoints. Table 1

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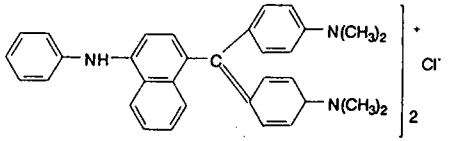
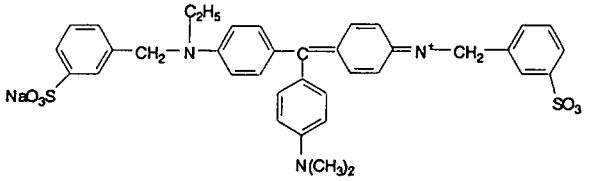
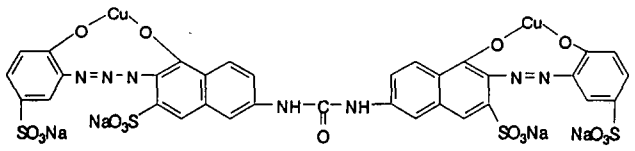
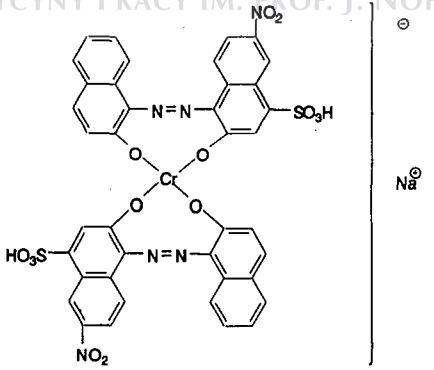
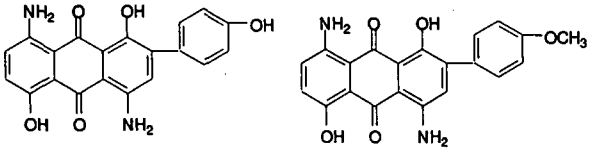
Table 1. The data of short-term tests for the studied dyes

Trade name, C.I. number	Structure, chemical class	Mutagenic and/or genotoxic profile	Effects	Relevant literature
Basic Blue 26, 44045	 Triphenylmethane dye	DNA damage, non-specific: peptide mutations in yeast (-S9)	+	Nagi (13) Combes et al. (6)
Acid Violet 49, 42640	 Triphenylmethane dye	*In vitro* host-mediated rec-assay, gene conversion in yeast.	–	Kada et al. (9) Combes et al. (6)
		Mutations in *S. typhimurium* (+S9)	+	Sugimura et al. (15); Bonin et al. (5) Combes et al. (6)
Direct Red 83, 29225	 Direct dye fast to light	Clastogenic: induction of micronuclei in bone marrow erythrocytes of mice;	–	Barański et al. (4)
		induction of sister chromated exchange in bone marrow and spleen cells of mice	–	

Acid Black 194	 Premetallized dye	Clastogenic: induction of microonuclei in bone marrow erythrocytes of mice	–	Barański et al. (4)
Disperse Blue 37	 Anthraquinone dye	Clastogenic: induction of micronuclei in bone marrow erythrocytes of mice;	–	Barański et al. (4)
		induction of sister chromatid exchange in bone marrow cells of mice	+	

– negative effect
+ poositive effect

gives the results of *in vivo* screening tests for three dyestuffs: Direct Red 83, Acid Black 194 and Disperse Blue 37, and the literature data on the effects of two triphenylmethane dyes: Basic Blue 26, and Acid Violet 49.

MATERIALS AND METHODS

Compounds

The following samples of dyes: Basic Blue 26, 44045, Acid Violet 49, 42640, Direct Red 83, 29225, Acid Black 194 and Disperse Blue 37 were obtained from Organika (Poland). They were used for external colouring only (of wool, jute, paper, natural silk, polyester, cellulosic fibres, biological preparations).

In the *in vitro* as well as *in vivo* tests, the dyes were assayd after they had been solved in sterilized distilled water on the day of the assay.

The median lethal dose (LD_{50}) for each sample was determined in a preliminary study, using the intraperitoneal exposure route according to the method of Weil (16).

All other chemicals and bacteriological media used in the tests were commercially available and of best quality.

In vitro test of *Salmonella typhimurium* cells

Mutagenicity assays were carried out using the standard top overlay agar plate assay described by Ames et al. (2) as revised by Maron and Ames (12). The *Salmonella typhimurium* his- strains (TA97a, TA98, TA100 and TA102) of different genetic specificity were used as targets of mutagenicity. They were obtained from Prof. Bruce N. Ames, University of California, Berkeley, CA.

Bacterial cells from stock cultures (maintained in frozen permanents at −80°C) were grown in liquid medium ("Oxoid" nutrient broth No 2) for 10h at 37°C with shaking.

The compounds were tested both in the absence and in the presence of metabolic activation (10% S9 fraction in S9-mix; 50 μl S9 per plate) which was obtained from the liver of Aroclor 1254-induced male stock Outbred Imp: Łódź rats. The amount of S9 protein per plate was 1.8 mg (in Basic Blue 26, Acid Violet 49, Direct Red 83, Acid Black 194 test) and 2.9 mg per plate (in Disperse Blue 37 test) for that particular batch of S9. Protein content was determined using the Lowry method (10).

The mutagenicity tests were performed in at least two series of assays with duplicate plates per dose; three plates were used for determinations of spontaneous reversion (negative controls). The assays were conducted to a cytotoxic and soluble concentration limit. To estimate the toxic effect, the spareness of the bacterial growth lawn was considered and the relative decline in the mutant values were determined.

The plates were incubated for 48 h at 37°C. Revertant colonies were counted with an automatic counter Biotran II from New Brunswick Scientific Co. Inc., NJ.

At least a 2-fold increase in the number of revertants per plate as compared to the number of revertants per control plates (number of spontaneous revertants) was the criterion for judging whether a given compound was a mutagen or not.

In vivo tests of bone marrow cells

Animals

Male and/or female mice were used at the age of 7 – 10 weeks, weighing between 23 and 30 g. The animals were fed with standard diet and water was given *ad libitum.*

All tests were performed on mice of Balb C strain, except Direct Red 83, 29225 which was given to SFISS strain when SCE analysis studies were performed. Four or five mice were used, each per dose for test compound, solvent and positive control.

Statistics

In the micronucleus and SCE assay, the statistical analysis of the results was performed using one-way analysis of variance with the multiple comparison tests (7).

Assay of micronucleus induction

Micronucleus assays were carried out using the method described by Schmid (14).

The frequency of micronucleated polychromatic erythrocytes in bone marrow cells of Balb C mice of both sexes after i.p. administration of test compounds was examined for two dose levels. In the first experiment series, the male mice were given doses equal to 80% LD_{50}. In the second series, the test compounds were given to male mice at 40 and 80% LD_{50} and female mice at 80% LD_{50}. The doses were divided in two equal parts and given in a volume 0.5 ml at 24 h intervals (after 24 h each mouse was given a second identical dose). The bone marrow cells and smears were prepared 30, 48 and 72 h after the administration of the first dose.

Micronuclei were identified as dark-blue-stained elements in the cytoplasm of the polychromatic erythrocytes (1000 per animal). As a measure of bone marrow cytotoxicity, the ratio of polychromatic to normochromatic cells was determined by counting 500 cells for each animal.

To elucidate clastogenic activities of test compound, a significant induction of micronuclei in bone marrow polychromatic erythrocytes of mice compared to the control group for at least one of the time points was essential.

Assay of sister chromatid exchange

Sister chromatid exchange (SCE) assays were carried out according to the GENE-TOX Program (11), using subcutaneous implantation of 5-bromodeoxyuridine (BrdU) tablets (1).

The incidence of SCE in bone marrow cells of the male mice after i.p. administration of the test compounds was analyzed for four dose levels following implantation of BrdU tablets. The males received the tested dyes once at 20, 40, 60 or 80% of the respective LD_{50} dose in a volume 0.5 ml per mouse.

Bone marrow cells mitosis was inhibited by i.p. administration of 3.3 mg/kg colchicine two hours before the animals were sacrificed by cervical dislocation. Air dry slides were made from bone marrow after the cells had been hypotonized and fixed. The cells were stained differentially according to the cytogenetic method of Antoshina and Porjadkova (3). To determine SCE frequency, 50 metaphase cells per mouse were analyzed.

To elucidate genotoxic activities of test compound, a significant induction of SCE compared to the control group for at least one of the dose levels was essential.

RESULTS AND CONCLUSIONS

The mutagenicity data of the *Salmonella* reversion assay for five dyes are summarized in Table 2. Of the dyes studied in TA97a, TA98, TA100 and TA102 *S. typhimurium* strains, Acid Violet 49 and Acid Black 194 revealed a positive mutagen response, whereas Basic Blue 26, Direct Red 83 and Disperse Blue 37 were negative.

Table 2. Induction of reverse mutations in the *Salmonella typhimurium* cells

Test compoounds Dose (μg) plate	Number of revertant colonies per plate ± SD			
	Salmonella typhimurium strains			
	TA97a	TA98	TA100	TA102
Basic Blue 26				
No S9 activation				
0[a]	107±13	21±5	134±13	247±21
1	103± 9	19±2	146±17	261±10
10	102±13	19±7	128±19	264±22
100	79± 6	15±2	149±13	231±14
500	Tox	Tox	129±10	226± 4
With S9 activation				
0[a]	112±11	29±3	138±16	315±27
1	112±11	29±5	146± 5	334±26
10	124±11	29±7	141± 7	334±26
100	121±10	31±3	145±19	363±18
500	113±29	31±5	149± 2	373±30
Acid Violet 49				
No S9 activation				
0[a]	107±13	21±5	134±13	247±21
10	100± 9	15±4	134±11	233±20
100	95±17	15±3	128±17	320± 9
1000	Tox	18±4	119±14	505±53[b]
5000	Tox	19±2	127± 9	489±80
With S9 activation				
0[a]	112±11	29± 3	138±16	315±27
10	120±19	34± 1	143±15	436±21
100	107±13	68± 8[b]	144± 9	436±21
1000	83±15	68±13[b]	133± 6	603±31
5000	Tox	22± 3	118±13	629±32
Direct Red 83				
No S9 activation				
0[a]	121±12	24±4	144±13	282±12
1	111± 7	20±3	147±21	267±12
10	122± 7	22±5	135±20	268± 8
100	123±18	19±4	128±36	281±11
1000	113± 3	17±4	120±21	299±28

Table 2. (continued)

With S9 activation				
0[a]	138±11	31±3	127±10	333±15
1	113±17	28±4	145±26	326± 6
10	113±20	26±5	148±18	326± 6
100	113±24	27±7	141±13	328±16
1000	111±18	23±9	112±22	332±35
Acid Black 194				
No S9 activation				
0[a]	121±12	24± 4	144±13	289±17
10	110±11	29± 4	143±14	305±28
100	142± 9	43± 9	147±28	332±41
500	198±37	77±11[b]	171±37	377±55
1000	239±54	112±24[b]	190±42	419±25
With S9 activation				
0[a]	130±14	31±3	127±10	330±18
10	118±15	33±4	158±14	368±18
100	134±11	38±3	152±17	368±14
500	151±26	53±3	150±17	377± 7
1000	161±22	54±9	158±29	420±45
Disperse Blue 37				
No S9 activation				
0[a]	113± 7	26±2	139± 9	335±33
10	105± 3	25±8	134±12	346±43
100	129± 4	25±4	136± 8	345±32
500	124±21	21±2	135± 9	369±35
5000	129±23	18±2	121±12	393±65
With S9 activation				
0[a]	129±13	36±6	132± 8	402±21
10	132± 6	33±8	141±10	433±26
100	134± 9	40±6	144±11	433±26
500	138±16	30±3	135± 9	425±31
5000	115±18	24±8	148±14	442±45
Positive controls:				
No S9 activation				
4-nitroo-o-phenylene-diamine 3.0	256±25 (114±12)[c]	357±28 (23±4)[c]		613±38 (305±1)[c]
Sodium azide 1.5			766±40 (130±10)[c]	
With S9 activation				
2-aminfluorene 5.0	675±25 (121±13)[c]	733±37 (26±5)[c]	730±40 (130±6)[c]	588±56 (276±19)[c]

Tox – toxic effects (background growth reduced; the frequency oof reversions less than 75% of spontaneous levels)

[a] – spontaneous reversion

[b] – positive response (reversion above 2 times background)

[c] – the control values

In our studies, doses of 100 and 1000 μg Acid Violet 49 per plate induced only an approx. 2-fold increase in revertant numbers. As compared to the spontaneous revertant number, the positive responses were obtained both in the strain which was sensitive to frameshift mutagens – TA98 in the presence of the S9 activation system, and in the strain which is particulary sensitive to oxidising and cross-linking mutagens – TA102 in the absence of S9 fraction (in a dose-dependent manner). At 1000 μg per plate Acid Violet 49 was toxic to the *S. typhimurium* strain TA97a as shown by reduced bacterial growth. However, it can be noticed that the toxic effects of Acid Violet 49 were quite different in the various strains, with TA97a being the most sensitive, followed by TA98 and TA100, with TA102 as the least sensitive (on the basis of absolute increase in revertant colonies over the background; more than a 75% decrease in revertant numbers compared to the spontaneous level).

Data obtained in mutagenicity tests demonstrated that Acid Black 194 had an increased number of revertants compared to spontaneous values in all of the test strains (a dose-dependent increase in revertants number), but positive response was obtained in strain TA98. The mutagenic effect was observed when TA98 had

Table 3. Induction of micronuclei in bone marrow cells of Balb C mice

Test compounds	Total dose mg/kg	Number of mice sex	Harvest time h	% of polychromatic erythrocytes with micronuclei ± SD		Ratio of polychromatic to normochromatic erythrocytes PCEs/NCEs	
				male	female	male	female
Distilled water (solvent control)	1 ml	8M 4F	48	0.15±0.05	0.20±0.08	0.69	0.91
Basic Blue 26	31.6	12M	30	0.22±0.05		0.51	
			48	0.15±0.10		0.54	
			72	0.15±0.06		0.46	
	56.8	12F	30		0.20±0.11		0.51[b]
			48		0.15±0.06		0.93
			72		0.17±0.09		0.76
	63.2	23M	30	0.20±0.09		0.79	
			48	0.16±0.07		0.63	
			72	0.18±0.07		0.54	
Distilled water	1 ml	8M 4F	48	0.12±0.08	0.12±0.12	1.39	1.34
Acid Violet 49	336.0	4M	30	0.12±0.09		1.16	
			48	0.10±0.14		1.53	
			72	0.13±0.12		1.37	
	672.0	8M 4F	30	0.14±0.09	0.10±0.08	0.87[a]	0.73[a]
			48	0.14±0.07	0.08±0.05	1.19	1.12
			72	0.14±0.09	0.15±0.05	1.04[b]	1.21
Mitomycin C (positive control)	2.5	4M	30	4.15±0.38[a]		0.71[a]	
			48	4.22±0.49[a]		0.48[a]	
			72	1.25±0.21[a]		0.14[a]	

a – $p < 0.01$; b – $p < 0.05$ – significant difference vs. control

been used in the absence of S9 fraction. With S9 fraction, the number of revertants was reduced in each test strains. Data show that Acid Black 194 at a dose of 500 μg per plate was able to induce an approx. 3.2-fold and at a dose of 1000 μg per plate an approx 4.7-fold increase in the number of revertants in strain TA98.

Table 3 shows the effects of i.p. administration of the two dyes, Basic Blue 26 and Acid Violet 49, on the formation of micronuclei in Balb C strain mice. Studied dyes did not increase, at any dose, the frequency of polychromatic erythrocytes containing micronuclei over control values of animals receiving distilled water only. Therefore, the dyes revealed neither clastogenic nor spindle-damage effect *in vivo*.

As expected, statistically significant increase in the frequency of micronucleated polychromatic erythrocytes was observed in the presence of control genotoxic agent mitomycin C. The finding demonstrates sufficient sensitivity of the assay system.

Decrease in the ratio of polychromatic erythrocytes to normochromatic erythrocytes is an indication of cytotoxicity of Basic Blue 26 and Acid Violet 49. Basic Blue 26 reveals toxic properties for erythrocytes of bone marrow of the female mice at 80% LD_{50} (56.8 mg/kg b.w.), while Acid Violet 49 for erythrocytes of the male and female mice at the same dose 80% LD_{50} (672.0 mg/kg b.w.).

Table 4 shows the effects of the three dyes, Basic Blue 26, Acid Violet 49 and Acid Black 194 on sister chromatid exchange (SCE) frequencies in bone marrow cells of mice. There no increase in SCE frequency at any dose resulting from administration of the two dyes, Basic Blue 26 and Acid Violet 49. Therefore, the dyes were incapable of inducing SCE in bone marrow cells. The mean frequency of SCE per cell for the Acid Black 194 was significantly different from that of the control ($p < 0.05$), showing clear dose-response relationship. All administrated doses of this dye increased SCE frequencies from 5.56 ± 0.24 per cell to 8.21 ± 0.77 and were significantly higher than that observed in the control group (3.95 ± 0.23). Therefore, Acid Black 194 has genotoxic effect on the bone marrow cells of mice.

From the results of the mutagenic and genotoxic tests with bacterial and mice bone marrow cells, one may conclude that:

1. Basic Blue 26, 44045 and Direct Red 83, 29225 dyes have no genotoxic properties as inferred from negative results in Ames, MN and SCE tests.

2. Acid Violet 49, 42640 and Disperse Blue 37 dye tests gave inadequate evidence for mutagenic/genotoxic activity as inferred from positive results in only one of three applied tests (Ames test for Acid Violet 49 and SCE test for Disperse Blue 37).

3. Acid Black 194 dye testing in a three-set battery of short-term assays gave a limited evidence for genetic activity (the positive results in Ames and SCE tests).

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Table 4. Induction of sister chromatid exchange in bone marrow cells of mice

Test compounds	Dose mg/kg	Number of mice	SCE/cell ± SD					Mean SCE/cell
Distilled water (solvent control)	0.5 ml	5	4.00±1.55	4.38±1.99	3.92±1.96	4.00±1.78	4.34±2.36	4.13±0.21
Basic Blue 26	15.80	5	4.34±2.10	5.02±2.39	4.70±2.10	4.50±2.17	4.86±2.37	4.69±0.30
	31.60	5	5.04±1.95	5.50±2.95	–	4.68±2.05	4.50±2.20	4.93±0.44
	47.40	5	4.10±2.04	4.54±1.93	4.26±1.69	4.12±1.77	3.96±1.94	4.20±0.22
	63.20	5	4.44±1.85	4.62±2.13	4.72±2.30	4.42±2.01	4.56±2.01	4.55±0.13
Distilled water	0.5 ml	4	4.06±1.89	4.02±1.86	4.12±1.93	3.94±1.86		4.04±0.08
Acid Violet 49	168.00	5	4.18±1.00	4.02±1.35	4.28±1.76	4.42±1.49	3.64±1.67	4.11±0.30
	336.00	5	3.50±1.74	4.08±1.68	4.28±1.87	4.12±2.11	4.84±2.11	4.16±0.48
	504.00	5	4.74±2.52	4.74±1.98	4.30±2.62	4.28±1.76	4.40±1.68	4.49±0.23
	672.00	5	4.32±1.85	4.74±1.98	4.58±1.86	4.66±2.33	4.66±2.14	4.59±0.16
Distilled water	0.5 ml	5	3.74±2.11	3.70±2.01	4.20±2.18	4.16±2.06	3.96±2.08	3.95±0.23
Acid Black 194	34.00	5	5.20±2.28	5.60±2.24	5.60±1.71	5.88±2.51	5.54±2.55	5.56±0.24[a]
	68.00	5	6.54±2.55	6.88±1.80	6.64±1.72	6.38±2.35	mouse died	6.61±0.21[a]
	102.00	5	7.46±3.14	7.36±3.26	7.00±2.74	7.34±2.37	7.10±2.31	7.25±0.19[a]
	136.00	5	9.14±2.81	8.90±3.09	7.96±3.19	7.42±2.56	7.64±2.84	8.21±0.77[a]
Mitomycin C	2.00	4	29.22±8.85	30.46±5.92	26.78±7.02	28.52±5.57		28.75±1.54[a]

[a] $p < 0.05$ significant difference vs. control

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