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GENOTOXICITY TESTING OF SOME DYES IN THE DIPLOID YEAST *SACCHAROMYCES CEREVISIAE*

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Ten synthetic colours were tested for the induction of mitotic gene conversion, mitotic crossing over and reverse mutation in the diploid strains D4 and D7 of *Saccharomyces cerevisiae*: Methyl Red, Orange G, Eriochrome Black T, Congo red, Rose Bengal, Fosin Y, Orange G, Phloxine B, Violet Acid 5B, Rouge S and Auramine O. All except rouge S and auramine O, failed to induce detectable genetic damage in yeast without metabolic activation. Auramine O and Rouge S induced significantly higher frequencies of mitotic recombinants and revertants in log-phase as well as in stationery-phase cells of the diploid yeast strains D4 and D7.

Key words: Mutagenicity, Recombinogenicity, Food colours.

Introduction

Short term *in vitro* tests are widely used to detect the mutagenic and recombinogenic potential of environmental chemicals. These tests for genotoxicity are used to predict the carcinogenic potential of chemicals. Their usefulness is based mainly on a fairly good correlation between mutagenicity and carcinogenicity of most chemicals [1].

In genotoxicity testing, a major focus has been on agricultural and industrial chemicals. However, these alone cannot be wholly responsible for human cancers. Naturally occurring or artificially added compounds ingested as part of the human diet have been related with incidence of certain cancers [2]. The present study was conducted to assess the mutagenic and recombinogenic potential of 10 synthetic dyes (mostly food dyes) currently or previously used all over the world. They were tested for the induction of mitotic gene conversion, crossing over, and reverse mutation in the diploid yeast, *Saccharomyces cerevisiae.*

Materials and Methods

The relevant information about the tested dyes is presented in Table 1. The dyes were dissolved in distilled water except Methyl Red, Eriochrome Black T, and Auramine O, which were dissolved in 50% ethyl alcohol. The test solutions were membrane filtered prior to use. Initial dose levels were orbitrarily choosen to be 1% and the dose was lowered if toxicity was observed.

The experimental methods have been described in detail by Chughtai *et al.* [3]. The yeast strain D7, described by Zimmermann *et al.* [4] was used for the simultaneous detection of mitotic crossing over at the *ade2*, mitotic gene conversion at the *TRP5*, and reverse mutation at the *ilvl* locus. The dyes were tested both with stationary and log-phase cells of the yeast. In stationary-phase tests, the cell suspensions having 10^7 cells/ml were treated with the test chemicals for 4 hr. at 30°C on a shaking reaction incubator. Parallel positive and negative control suspensions containing ethyl methane sulphate (EMS, a well known mutagen and recombinogen) and distilled water respectively, were also prepared. The cells were harvested by centrifugation for dilution and plating. About 10^6 cells from each suspension were plated onto the appropriate synthetic minimal media lacking tryptophan and isoleucine for scoring of revertants and convertants, respectively. The strain D7 requires tryptophan and isoleucine-valine for growth but no adenine. Mitotic gene conversion in D7 was monitored by the appearance of tryptophan non-requiring colonies on selective synthetic minimal medium lacking tryptophan. The alleles involved (*TRP5-12* and *TRP5-27*) have been derived from the widely used strain D4 [4,5]. D7 is homoalellic at the *ilv1* locus *(ilv1-92/ilv1-92)*. The isoleucine requirement can be alleviated by true reverse mutation and allele non-specific suppressor mutation. About 100-200 cells were plated per YEPD (complete medium containing yeast extract, peptone and dextrose) plate for survival estimation and visual scoring of mitotic crossover events (The red/pink twin-sectored colonies). These are formed due to the homozygous cells of the genotype *ade2-40/ade2-40* (deep red) and *ade2-119/ade2-119* (pink) [4]. The *ade-2* locus of D7 has been derived from the strain D5. Various other types of aberrant colonies result from the mutagenic treatment of D7. These colonies are whole red, whole pink, red/white sectored, pink/white sectored and hairlines. The aberrant colonies could result from either mitotic recombination, mutation or chromosomal aberrations [4].

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Treatments in log-phase were given as described by Sankaranarayanan and Murthy [6]. About 200-300 cells/5ml of liquid YEPD were inoculated from a culture of D7 with low rate of spontaneous conversions and reversion. After 24 hr. of growth, the test solution was added and the treatment continued in dark on a shaking reaction incubator at 30°C. After 48 hr. (i.e. 24 hr. after addition of the test chemicals), the cells were harvested by centrifugation, counted by haemocytometer, diluted and plated onto the apropriate media for estimating rate of cell survival, conversion, and reversion.

Two dyes, Rouge S and Auramine O, were also tested in the growing cells of another diploid yeast strain D4, which is heteroallelic at two unlinked loci, *ade2 (ade2-2/ade2-1)* and *trp5 (trp5-12/trp5-27)* [5]. The alleles at both these loci are non-complementing resulting in requirement for adenine and tryptophan for growth on minimal media. The occurrence of mitotic gene conversion at these loci creates cells no longer requiring either adenine or tryptophan. Thus, the convertants can be easily obtained by selective plating technique [5]. All plates were incubated at 30°C in the dark, and the colonies counted after 72 and then after 120 hr. of incubation.

Results and Discussion

Results on the genotoxicity of the tested dyes are presented in Table 2-4. These results indicate that eight of the dyes did not induce mitotic gene conversion, mitotic crossing over and reverse mutation in both stationary and log-phase cells of S. *cerevisiae* strain D7 without any further metabolic activation. Only two of the dyes, Auramine O and Rouge S, proved potent mutagens and recombinogens in strains D4 and D7 under the experimental conditions employed. Unlike the non-mutagenic dyes, they both significantly reduced cells viability (survival) and inhibited cell division (indicated by reduced cell count at the end of the treatments in log-phase tests). Seven of these dyes (Methyl Red, Eriochrome Black T, Congo Red, Rose Bengal, Eosin Y, Orange G and Violet Acid 5B) were tested in strain D4 of S. *cerevisiae* with negative results (B. A. Siddiqui and S. R. Chughtai, unpublished data). As far as their human use is concerned, three of the tested dyes, that is, Orange G, rose Bengal and Violet Acid 5B, had restricted use in foods, Congo Red has been delisted, Phloxine has been widely used in drugs and cosmetics, while Eosin Y was widely used in foods according to a 1975 report [7].

The inactivity of Methyl Red and Eriochrome Black T in the present study may be due to their requirement of metabolic activation for mutagenicity in Ames test[8,9]. Methyl Red also failed to induce chromosome damage in chinese hamster ovary (CHO) cells [10]. Orange G was negative in the Ames [11], *E. colifluctuation* [12], *Drosophila* sex-linked recessive lethal tests and in plant cells [13]. There is one report on its clastogenicity in CHO cells [10].

Congo Red was Ames negative [14] but was active in this test following preincubation with rihoflavin-rich S9 mix [9]. However, Robertson *et al.* [15] were unable to detect its

Table 1. Background Information about the Tested Colours.

Trival names	FD & C or C. I. name	C.I. number	Chemical class	Manufacturer's name and address	Source
Methyl Red	Acid Red 2	13020	Monoazo	BDH Chemical Ltd., Pool, England	Purchased from manufacturers
Rose Bengal	Food Red 93	45435	Yanthene	E. Merck Darmstadt, W. Germany	"
Orange G	Food Orange 4	16230	Monoazo	E. Merck Darmstadt, W. Germany	"
Eosin Y	D & C Red 22 (Acid Red 87)	45380	Xanthene	E. Merck Darmstadt, W. Germany	"
Phloxine B	D & C Red 28 (acid Red 98)	45405	Xanthene	E. Merck Darmstadt, W. Germany	"
Eriochrome Black T	—	14645	Monoazo	E. Merck Darmstadt, W. Germany	"
Congo Red	Direct Red 2	22120	Diazo	P.P.H. Polskies, Poland	"
Acid Violet 5B	F & C Violet 1 (Benzyl Violet 4B)	42640	Triphenyl methane	Paul Entrop, Emmanvellaan, Belgium	Manufacturer's gift
Rouge S	—	—	—	"	"
Auramine O	Basic Yelow 2	41000	Xanthene	Ciba Ltd., Balse, Swit .erland	Purchased from local market.

A=Rouge S and acid violet 5B were of the food grade quality while the rest of the dyes were laboratory grade since they are also used as biological stains. The purity of Auramine O is not certain since it was purchased from the local market and tested without purification. Its use in Pakistan as a food colouring is elicit since the manufacturer is not a known food dye manufacturer.

TABLE 2. PERCENT SURVIVAL AND FREQUENCY OF CONVERTANTS (Trp+) AND REVERTANTS (Ilv+) in *S. cerevisiae* STRAIN D7.

Test compound	Concentration[A] (mg/ml)	Survival[A] %	Cell count (cells/ml x10^8) in log-phase teste	Frequency per 10^6 survivors[B]			
				Log-phase tests		Stationary-phase tests	
				Trp+	Ilv+	Trp+	Ilv+
Water (negative control)		100[100]	3.50	70.25	3.25	B	B
EMS (positive control)	0.01ml/ml	99[95]	3.55	670.66**	90.35	234**(24)	158**(20)
Methyl Red[C]	1.00	98	–	–	–	10(11)	12(21)
Orange G	10.00	100	–	–	–	18(20)	10(9)
Eriochrome Black T[C]	1.00	99	–	–	–	8(51)	19(17)
Acid Violet 5B	1.00	95	–	–	–	15(14)	5(6)
Congo Red	1[1]	100[97]	3.35	60.00	2.00	63(60)	5(3)
Rose Bengal	1[1]	99[100]	3.40	63.75	4.10	51(48)	5(4)
Eosin Y	10[1]	98[99]	3.45	76.33	4.50	62(50)	4(3)
Phloxine B	1[1]	99[98]	3.75	63.75	3.20	44(48)	3(3)
Auramine O[C]	0.05[0.05]	60**[40]**	3.00**	265.85**	18.37**	796(70)**	88(6)**
	[0.10]	[24]**	1.75**	285.00**	26.62**		
Rouge S	0.20[0.10]	50**[64]**	1.75**	280.00**	25.75**	691(32)**	262(8)**

A =Concentrations and % survival given in brackets are for the log-phase tests. These tests had a common negative control for comparison.
B =Frequencies are the means from 4 plates. The values in parentheses are for the respective negative controls in stationary phase tests.
** =Differ significantly (x^2 test) from the respective negative control values.
C =These dyes were dissolved in 50% ethyl alchol and had the same solution as negative control.

TABLE 3. FREQUENCY OF MORPHOLOGICALLY ABERRANT COLONIES IN *S. cerevisiae* STRAIN D7.

Test	Test Compound[A]	Number of colonies examined	Total number of aberrant colonies	Aberrant colonies/10^3 survivors	Number of Twin-sectored colonies	Twin-sectored colonies/10^3 survivors
Stationary Phase						
	EMS (positive control)	8700(10800)	800(75)	91.95(6.9)**	68(3)**	7.82**(0.28)
	Methyl Red	8600(10600)	51(62)	5.93(5.8)	3(3)	0.35(0.28)
	Orange G	1200(6825)	54(26)	4.50(3.8)	2(1)	0.16(0.15)
	Eriochrome Black T	11250(17000)	60(75)	5.89(4.4)	3(4)	0.27(0.24)
	Congo Red	15400(17500)	60(66)	3.89(3.8)	3(2)	0.19(0.12)
	Rose Bengal	13350(16675)	29(42)	2.17(2.5)	2(2)	0.15(0.12)
	Eosin Y	8700(9700)	40(49)	4.59(5.1)	2(2)	0.22(0.21)
	Phloxine B	12500(13200)	58(60)	4.64(4.6)	2(2)	0.16(0.15)
	Acid Voilet 5B	15500(18000)	30(34)	1.93(1.9)	2(2)	0.13(0.11)
	Rouge S	6400(12250)	270(50)	42.18(4.1)**	14(2)**	2.19**(0.16)
	Auramine O	13000(12750)	85(22)	6.54(1.7)**	10(2)**	0.76**(0.16)
Log Phase						
	EMS(positive control)	12200(11750)	819(88)	66.39(7.5)	76(3)**	6.23**(0.26)
	Congo Red	17900(16600)	53(54)	2.96(3.3)	3(2)	0.17(0.12)
	Rose Bengal	13250(14250)	29(28)	2.19(1.9)	2(3)	0.15(0.21)
	Eosin Y	17500(16900)	42(44)	2.40(2.6)	2(2)	0.11(0.12)
	Phloxine B	15500(16350)	46(47)	2.97(2.9)	3(2)	0.19(0.12)
	Rouge S	12000(12500)	140(24)	11.66(1.9)**	15(2)**	1.25**(0.16)
	Auramine O	17600(18275)	230(53)	13.07(2.9)**	25(3)**	1.42**(0.16)

** =Differ significantly from the respective negative control values given in parentheses (P>0.01). A =Concentrations are as in Table 2.

TABLE 4. FREQUENCY OF MITOTIC GENE CONVERSION AT THE ADE2 AND TRP5 LOCI IN LOG-PHASE TESTS OF *S. CEREVISIAE* STRAIN D4.

Test compound	Concentration (mg/ml)	Cell count (cells/mlx10^8)	Survival %	Convertants per 10^6 survivors Ade$^+$	Trp$^+$
Rouge S	0 (negatic control)	1.60	100.00	16.80	7.50
	0.01	1.30**	98.70	36.45	24.00**
	0.05	1.10**	45.00**	70.30**	60.15**
	0.10	0.85**	36.10**	93.20**	76.20**
	0.2	0.43**	26.00**	115.00**	90.00**
	0.5	0.10**	14.00**	126.80**	105.60**
Auramine O	0 (negative control)	2.50	100.00	11.20	3.20
	0.01	1.37**	85.0C**	26.70**	5.70
	0.05	1.00**	61.87**	45.60**	18.90**
	0.10	0.50**	47.00**	52.50**	55.30**
	0.2	0.05**	11.50**	64.00**	86.00**
	0.5	0.03**	5.00**	74.00**	97.00**

** Differ significantly from the negative control values.

activity in this test using preincubation with S9 mix supplemented with ATP, NADH and riboflavin, but supplementation with FAM and FAD facilitated the detection of mutagenicity. Thus the inactivity in the present report and in an earlier yeast gene conversion test [16] may be due to lack of activation in this organism.

Rose Bengal was active in several mutagenicity tests with bacteria [17-19]. It was, however, negative in Ames and clastogenicity assays but active in rec-assay without microsomal activation [20,21]. It also induced chromosomal damage in CHO cells [10,22] but was inactive in an earlier gene conversion test with *S. cerevisiae* after light activation [23].

Eosin Y induced chromosomal abnormalities in *Vicia faba* but not in *Allium cepa* [13] and after light activation induced mutations in Ames and rec-assays [18] and inactivated the transforming DNA [19]. The dye has, however, been reported as negative in Ames test [24], rec-assay [16] CHO cells [10] and yeast gene conversion tests [23,25].

Phloxine was active in *E. coli* rec- and pol-assays [12,26] but initial indications of its mutagenicity in *F. coli* [17] were not confirmed in more sensitive fluctuation tests [12,24]. Kawachi *et al.* [21] and Ishidate [20] reported that phloxine was inactive in ames and clastogenicity tests but was active in rec-assay only in the absence of activation. The dye induced mutations in human lymphocytes [27] while the mutagenicity of certain lipstick formulations was attributed to their phloxine content [18]. The dye induced light dependent inactivation of transforming DNA and rec-assay positive effects [19] but Haveland-Smith *et al.* [26] found no evidence for mutation or photoactivation. The inactivity of phloxine in the present study is consistent with earlier gene conversion tests with yeast [16,28]. The food dye violet acid 5B was inactive in rec-assay [17] and Ames test [29]; the only two reported studies.

Auramine O, detected as a recombinogen and mutagen in the present study, induced mitotic crossing over in other yeast strains [30-32] was active in *F. coli* pol-assay but failed to induce reverse mutation in S.*typhimurium* [33].

Rouge S, a red food dye, was detected as a potent mutagen and recombinogen in the present study with diploid yeast since the information about its chemical composition, structure and carcinogenic potential is not available, and since the dye has not been tested for mutagenicity elsewhere, it is not possible to make further comments about its genotoxic status.

For the genotoxicity evaluation of the food dyes, the metabolizing potential of the yeast organism must be given due consideration. Thus the observation that *S. cerevisiae* and other microbial systems reduced several dyes may be important [26,28]. It is also important that all of the dyes reported as negative in earlier yeast gene conversion tests (Congo Red, Rose Bengal, Fosin Y. Phloxine) were inactive while Auramine O, the only dye with positive effects in other strains was detected as recombinogen in the present studies.

The conflicting and equivocal data about the genotoxicity could be attributed to various factors; the important ones being the organismal specificities and activation or inactivation by the metabolizing systems, the repair mechanisms, sample variations, different known or unknown mutagenic or nonmutagenic impurities, and the requirement for photoactivation of the xanthene dyes. We are aware that these preparations may contain impurities. However, no further purification was attempted because we wanted to assess the potential danger that they pose in actual use.

In view of the conflicting data about the genotoxicity of most of the dyes tested in the present study and the large extent of the human population exposed, further testing is essential.

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