

ASSESSMENT OF THE GENOTOXIC POTENTIAL OF CAMEL COLOUR I IN FOUR SHORT-TERM TESTS

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Abstract—A battery of three short-term tests *in vitro* and one *in vivo* was used to determine the genotoxicity of Caramel Colour I. The results of the bacterial mutation assay, using five strains of *Salmonella typhimurium*, and the mouse micronucleus assay *in vivo* showed no evidence of genotoxic activity. Results from both the cytogenetics assay *in vitro*, using CHO cells, and the mouse lymphoma assay indicated that there was some genotoxic activity associated with Caramel Colour I but only in the absence of S-9 and at very high dose levels.

INTRODUCTION

Caramel Colour I is produced by the controlled heating of carbohydrates in the presence of an acid or an alkali. The details of the methodology are discussed in more detail elsewhere in accompanying papers within this special issue. This class of caramel colour, together with Classes II, III and IV, has been the subject of a number of short-term testing programmes in the past which, in general, have generated negative results. Some weakly positive data (Aeschbacher, 1986; Ishidate *et al.*, 1984; Jensen *et al.*, 1983; Kawachi *et al.*, 1980; Yu *et al.*, 1984) had, however, been generated and in response to this the International Technical Caramel Association (ITCA) initiated a comprehensive testing programme, of which these studies are a part.

MATERIALS AND METHODS

Test sample

The test sample (batch number ETA-128-1F), in the form of a brown viscous liquid, was supplied by ITCA. Details of the analytical data pertaining to the sample are given in Table 1. More details of preparative methods and comparison with other caramel colours can be found in accompanying papers (Allen *et al.*, 1992; Brusick *et al.*, 1992).

Chemicals

Positive control compounds for the tests *in vitro* in the absence of S-9 mix were as follows:

Bacterial mutation assays: 2-nitrofluorene (TA98, 1 µg/plate; TA1538, 2 µg/plate); *N*-ethyl-*N*-nitro-

N-nitrosoguanidine (TA100, 3 µg/plate; TA1535, 5 µg/plate); 9-aminoacridine (TA1537, 80 µg/plate).

Cytogenetics assay *in vitro*: mitomycin C at a final concentration of 0.2 µg/ml.

Mammalian cell mutation assay: ethylmethanesulphonate at a final concentration of 500 µg/ml.

Positive control compounds for the tests *in vitro* in the presence of S-9 were as follows:

Bacterial mutation assays: 2-aminoanthracene at 0.5 µg/plate (TA98, TA1538), 1 µg/plate (TA100) or 2 µg/plate (TA1535, TA1537).

Cytogenetics assay *in vitro*: Cyclophosphamide at final concentrations of 15 and 20 µg/ml.

Mammalian cell mutation assay: 20-methylcholanthrene at a final concentration of 2.5 µg/ml.

Mitomycin C was also used as the positive control for the mouse micronucleus test *in vivo*.

Bacterial mutation test

The method used is based on that published by Ames *et al.* (1975) and Maron and Ames (1983) using five strains of *Salmonella typhimurium* (TA1535, TA1537, TA1538, TA98 and TA100), kindly supplied by Dr B. Ames. Briefly, aliquots of bacterial suspension and sodium orthophosphate buffer were added to five concentrations of Caramel Colour I. The concentrations used were separated by approximately half-log₁₀ intervals with a top dose of 10 000 µg/ml. The appropriate positive and negative controls were set up in a similar manner. These treatments were then mixed with histidine-deficient agar and overlaid on to previously prepared plates containing 25 ml of minimal agar. The plates were then incubated for 3 days and the colonies growing on the plates counted using an electronic colony counter. The test was repeated to confirm the results obtained.

Cytogenetics assay *in vitro*

Chinese hamster ovary (CHO) cells, strain K₁BH₄, originally obtained from BIBRA Toxicology

Abbreviations: CHO = Chinese hamster ovary; CP = cyclophosphamide; DHS = donor horse serum; FCS = foetal calf serum; ITCA = International Technical Caramel Association; MI = mitotic index; MMC = mitomycin C; TFT = trifluorothymidine.

Table 1. Analytical data for Caramel Colour I

Analytical data	Batch No: ETA-128-IF
Colour intensity, K_{610} *	0.1
Solids (%)	70.0
Nitrogen (%)	0.01
Sulphur (%)	0.03
Ammoniacal nitrogen (%)	<0.005†
Sulphur dioxide (%)	<0.005†
Ash (%)	1.0
4-Methylimidazole (ppm)	<5†
Arsenic (ppm)	<1†
Lead (ppm)	<1†
Mercury (ppm)	<0.1†

*Absorbance at 610 nm, path length 1 cm, of an aqueous solution of 1 g caramel colour solids/litre.

†Below the limit of detection.

International, were routinely subcultured in Ham's F12 medium supplemented with 5% foetal calf serum (FCS) at 37°C in a humidified atmosphere containing 5% CO₂. 24 hr before treatment, cultures were established by seeding approx. 4×10^5 cells in 5 ml medium per tissue culture flask (Nunc) and incubating as above. At treatment time, 1.25 µl S-9 mix was added to one set of cultures followed by 62.5 µl of the various dilutions of test compound, positive control or solvent. 50 µl of the test compound, positive control or solvent were added to the second set of flasks (i.e. without S-9 mix) to give the required final concentrations. Two flasks were prepared for each concentration of the test compound and for the positive controls. Four flasks were prepared from each solvent control. 4 hr after addition of test compound to those cultures containing S-9, the medium was carefully removed and replaced with fresh Ham's plus 5% FCS and the cultures returned to the incubator. 19 hr after the initial treatment, all cultures were treated with colchicine (final concentration 0.25 µg/ml) and returned to the incubator for a further 2 hr. Cells from each culture were then fixed with methanol-acetic acid (3:1), dropped on to cold, precleaned slides and stained with 10% Giemsa. After air-drying, slides were mounted in DPX. The incidence of mitotic cells/1000 randomly scored cells was assessed for each culture. When expressed as a percentage this value is known as the mitotic index (MI). The cultures selected for full metaphase analysis were chosen on the basis of the MI information. Slides from these cultures were coded, examined microscopically and, when possible, 100 metaphases from each culture scored for chromosomal aberrations.

Cell mutation assay

Mouse lymphoma L5178Y cells, obtained from Dr J. Cole, Sussex University, were routinely cultured as suspensions in RPMI 1640 (Imperial Laboratories) supplemented with sodium pyruvate (110 µg/ml), Synperonic F68 (1 mg/ml), gentamicin (50 µg/ml) and 10% heat-inactivated donor horse serum (DHS Imperial Laboratories). Before treatment, 12-ml aliquots of cell suspension at 1×10^6 cells/ml were set up to which were added 8 ml S-9 mix or RPMI 1640.

200 µl test material, solvent control or positive controls were then added. Two cell cultures were prepared for each dose. Treated cultures were then placed on a roller apparatus for 3 hr at 37°C, after which the cells were pelleted by low-speed centrifugation, washed with RPMI containing 10% DHS and transferred to a roller flask containing 60 ml growth medium. The cells were maintained in suspension culture for 48 hr to allow for expression of induced mutation. During this period relative total growth was measured by analysing the cell density of each treated culture after 24 and 48 hr and comparing this with the cell density of the untreated controls. At the end of the 48-hr incubation period, the cells were cloned in soft agar to permit measurement of levels of viability and induced mutation. Cells were plated, in triplicate for each treatment, at approximately 200 cells/90 mm dish and 10⁶ cells/90 mm dish to assess viability and mutant frequency, respectively. The selective agent, trifluorothymidine (TFT) at a final concentration of 4 µg/ml was also incorporated into the mutation plates. The plates were then incubated at 37°C in an atmosphere of 5% CO₂ in air for 12 days. Colonies growing on the plates were then counted using an electronic colony counter.

Mouse micronucleus test

CD-1 mice (Charles River, UK), approx. 40 days old, were used for the test. The mice were tail-marked for identification and fed a commercial diet (Labsure LAD 1) and offered water *ad lib*. In a preliminary dose-range-finding study, no adverse clinical signs were seen and a limit dose of 5000 mg/kg body weight was chosen for the micronucleus test. 15 males and 15 females were dosed with either the vehicle control or test solution; five animals of each sex were dosed with the positive controls. All animals in all groups were given a single oral dose by intragastric gavage using a standard volume of 20 ml/kg body weight. Following dosage, animals were examined regularly and any deaths or clinical signs recorded. Five males and five females from the vehicle control and test solution groups were killed at 24, 48, 72 hr after dosing. All animals from the positive control group were killed at 24 hr after dosing. Femurs were cleared of tissue, and direct bone marrow smears made on to slides following the removal of an epiphysis. One smear was made per femur and fixed in methanol for at least 10 min. Air-dried smears were then stained with Giemsa for 10 min [10% Gurr's R66 (BDH) in distilled water]. Following rinsing in distilled water and differentiation in buffered distilled water (pH 6.8), the smears were air-dried and mounted with coverslips using DPX (Proudlock and Allen, 1985).

Coded smears were examined, and at least 1000 erythrocytes scored per animal to determine the incidence of micronucleated polychromatic erythrocytes and the ratio of polychromatic to normochromatic erythrocytes. The number of micronucleated

Table 2. Summary of results of bacterial mutation tests on Caramel Colour I

Test no.	Sample	Dose level ($\mu\text{g}/\text{plate}$)	Mean relative colony counts*									
			TA1535		TA1537		TA1538		TA98		TA100	
			-	S-9†	+	-	S-9	+	-	S-9	+	-
1	Caramel Colour I	10,000	7	11	10	10	10	11	23	20	118	110
		3000	11	10	15	12	10	12	25	26	109	105
		1000	8	11	15	13	7	9	18	20	102	105
		300	12	12	12	9	10	11	29	22	109	105
		100	7	12	10	10	11	10	17	21	116	114
		0	7	10	13	11	9	13	23	18	113	108
		Solvent	9	10	9	12	10	11	23	19	114	104
2	Caramel Colour I	10,000	9	7	17	13	9	13	30	25	142	147
		3000	14	9	12	12	16	13	36	22	125	133
		1000	13	12	14	16	13	9	32	22	113	124
		300	14	12	15	15	8	14	29	21	146	123
		100	13	6	14	13	12	16	28	23	117	126
		0	11	9	10	16	5	11	28	16	108	103
		Solvent	11	11	15	13	14	13	32	22	123	115
Positive control	503	137	1872	163	49	217	76	189	342	732		

*Each value is the mean of triplicate plates.

†Present (+) or absent (-).

Table 3. Summary of results of micronucleus test on Caramel Colour I

Kill time (hr)	Compound	Dosage (mg/kg)	p:n ratio		Incidence mnp		Incidence mnn (total/1000)
			Mean	P	Mean/1000	P	
24	Vehicle	—	0.732	—	0.5	—	0.3
	Caramel	5000	0.708	0.485	0.4	0.537	0.0
	Colour I	12	0.557	0.140	40.5	<0.001	0.3
48	Vehicle	—	0.969	—	1.0	—	0.0
	Caramel	5000	0.785	0.062	0.8	0.530	0.0
	Colour I	5000	0.785	0.062	0.8	0.530	0.0
72	Vehicle	—	0.989	—	0.4	—	0.0
	Caramel	5000	1.138	0.711	0.5	0.588	0.2
	Colour I	5000	1.138	0.711	0.5	0.588	0.2

P = Results of statistical analysis using Wilcoxon's sum of ranks test (one-sided probabilities) p:n = ratio of polychromatic to normochromatic erythrocytes mnp = number of micronucleated polychromatic erythrocytes mnn = number of micronucleated normochromatic erythrocytes MMC = mitomycin C

normochromatic erythrocytes was also recorded as recommended by Schmid (1975).

RESULTS

Bacterial mutation test

Caramel Colour I was tested in Salmonella strains TA1535, TA1537, TA1538, TA98 and TA100 in the presence and absence of metabolic activation. Concentrations of Caramel Colour I up to 10,000 $\mu\text{g}/\text{plate}$ were used in all tests. No increases in the number of relevant colonies were observed in any of the tests carried out; the data are summarized in Table 2.

Micronucleus test

No evidence of genotoxic activity was found in the micronucleus test following treatment with Caramel Colour I, nor was there any evidence of bone marrow toxicity as determined by the ratio of polychromatic to normochromatic erythrocytes. The results of this test are summarized in Table 3.

Cytogenetics assay in vitro

In the absence of metabolic activation, the highest dose of Caramel Colour I, 10,000 $\mu\text{g}/\text{ml}$,

caused a highly significant increase in chromosomal damage; no significant increases in chromosomal damage were seen at any lower doses used either in the presence or absence of S-9. Analysis of the MI of cultures showed that no concentration of Caramel Colour I reduced the MI to less than 62.5% of the solvent control; it was therefore possible to examine metaphases from the top three treatment groups. The results are summarized in Tables 4 and 5.

Table 4. Mitotic indices (MI) of cultured Chinese hamster ovary (CHO) cells treated with Caramel Colour I

Caramel Colour I concentration ($\mu\text{g}/\text{ml}$)	MI	
	S-9 mix absent	S-9 mix present
0 (solvent control)	5.2	6.4
19.5	4.8	5.8
39	4.0	5.7
78	3.9	5.4
156	4.1	6.2
313	4.8	5.7
625	4.7	5.3
1250	4.6	5.3
2500	3.9	6.1
5000	4.6	4.0
10,000	4.1	4.2

Table 5. Effects of Caramel Colour I on the chromosomes of CHO cells

Test agent	S-9†	Concentration (µg/ml)	No. of cells examined	Aberrations‡ (no./100 cells)										No. of aberrant cells							
				-gaps	+gaps	BWF	BF	C	I	SM	A	GT	P	ISO	CHR	-gaps	+gaps	mean (%)	mean (%)		
Solvent	-	10 µl/ml	400	0.75	1.75	1					2							3	0.75	6	1.50
Control	-	1250.0	200	2.5	3					4	1							5	2.50	6	3.00
Caramel	-	5000.0	200	1	1					1	1							2	1.00	2	1.00
Colour I	-	10,000.0	200	12	15.5	6				1	6	11						21	10.50***	27	13.50***
MMC	-	0.2	200	58	61.5	14	1			25	27	40						59	29.50***	60	30.00***
Solvent	+	10 µl/ml	400	6.25	7.75	3				1	12	9						21	5.25	23	5.75
Control	+	1250.0	200	2	2	1				2	1							3	1.50	3	1.50
Caramel	+	5000.0	200	5.50	6	2				4	4	5						10	5.00	11	5.50
Colour I	+	10,000.0	200	4	4	1				1	3	3						8	4.00	8	4.00
CP	+	20.0	107	91.45	94.20	8	1			11	42	29						52	48.60***	52	48.60***

CP = cyclophosphamide MMC = mitomycin C

†Present (+) or absent (-).

‡BWF = Chromatid break with fragment; I = interchange; P = pulverised cell; A = acentric fragment; SM = single minute, BF = chromatid break without fragment; C = complex rearrangement; GT = greater than 10 aberrations; ISO = isochromatid gap; CHR = chromatid gap.

Statistical analysis used was Fisher's test. ***P < 0.001, otherwise P < 0.05.

Cell mutation assay

In the absence of metabolic activation, Caramel Colour I was tested at concentrations up to 8000 µg/ml in two independent tests. These top doses resulted in cell survival levels of 9 and 5% with respect to the solvent controls. Dose-related and statistically significant increases in mutant frequency were observed in both tests. It has been reported that extremes of pH and osmolality may cause increases in mutant frequency (Cifone *et al.*, 1987; Galloway *et al.*, 1987); accordingly, both these parameters were checked and found to be well within acceptable levels (data not given).

In the presence of metabolic activation, Caramel Colour I proved to be much less toxic than was the case with no activation. Cell survival at the highest dose of 9000 µg/ml was 59% relative to the control. In the first test no significant increases in the number of TFT-resistant colonies were observed, but in the repeat test small increases were observed. The largest increase, however, was only 1.55 times above the background level and was just outside the upper end of the historical control range. These results were considered to demonstrate that the inclusion of S-9 reduced or removed the genotoxic activity of the caramel colour. The data for these tests are summarized in Table 6.

DISCUSSION

The results from this and the accompanying papers by Allen *et al.* (1992) and Brusick *et al.* (1992) all indicate that none of the classes of caramel colours induces mutations in Salmonella. These results are consistent with the majority of the results reported previously for mutation and DNA repair tests in bacteria (Aeschbacher *et al.*, 1981; Ashoor and Monte, 1983; Bonin and Baker, 1980; Brusick, 1974; Kawana *et al.*, 1980) and the larger number of unpublished reports previously submitted to WHO and reviewed in the monograph produced from the 29th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1987).

Some positive results have been reported in the literature (Aeschbacher, 1986; Ishidate *et al.*, 1984; Jensen *et al.*, 1983; Kawachi *et al.*, 1980; Yu *et al.*, 1984). The mutagenic response in all cases was weak and, where such data were reported, completely abolished in the presence of metabolic activation (Aeschbacher, 1986).

No previous reports have been published dealing with the mutagenicity or clastogenicity of Caramel Colour I in cultured mammalian cells. The results of this study show that some evidence of mutagenic activity could be observed but only at very high doses, and that any effect was nullified by the presence of a metabolic activation system. Data showing weak genotoxic effects in mammalian cell lines have been reported for other caramel colours. These

Table 6. Summary of results from mammalian cell mutation tests for Caramel Colour I

Caramel Colour I dose ($\mu\text{g/ml}$)	S-9†	Test 1			Test 2		
		Mean survival (%)‡	Mean mutant colonies per plate	Mutant frequency§	Mean survival (%)‡	Mean mutant colonies per plate	Mutant frequency
0	—	100	37	47	100	40	67
1250	—	110	44	45			
2500	—				96	47	73
6000	—	33	79	115**			
6500	—				19	133	278***
7000	—	19	125	215***	25	160	343***
7500	—				12	117	312***
8000	—	9	111	217***	5	112	378***
Positive control	—	53	350	535***	80	301	656***
0	+	100	43	64	100	42	66
2500	+				104	43	63
4000	+	107	39	56			
6000	+	56	54	81			
6500	+	59	42	66			
7000	+	57	58	74			
7500	+				67	59	85*
8000	+				68	67	97**
9000	+				58	62	101**
Positive control	+	70	228	320***	61	183	441

†Present (+) or absent (—)

‡Mean survival (%) = survival relative to negative control.

§Mean mutant frequency = mutants/ 10^6 survivors.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

include the induction of sister chromatid exchange in diploid human fibroblasts (Sasaki *et al.*, 1980), chromosome aberrations in Chinese hamster lung cells (Ishidate *et al.*, 1984), chromosome aberrations in Chinese hamster ovary cells (Stich *et al.*, 1981), and unscheduled DNA synthesis in human cells (Yu *et al.*, 1984). Again, a common finding of these reports was that the presence of S-9 reduced or completely abolished any genotoxic effect.

The negative result of the micronucleus test *in vivo* confirms the results in the studies *in vitro* in which positive effects are nullified by the presence of mammalian metabolic enzyme systems. Other reports with a range of caramel colours also showed no evidence of genotoxicity *in vivo* (Kawachi *et al.*, 1980) or carcinogenicity in mice (Hagiwara *et al.*, 1983) and rats (Maekawa *et al.*, 1983).

Conclusion

The overall conclusion of the data reported here is that Caramel Colour I is non-genotoxic in the bacterial mutation assay and mouse micronucleus test. There is some evidence for genotoxicity in the cytogenetics assay and the mouse lymphoma assay. However, this evidence is found only at high doses in tests *in vitro* where no exogenous metabolic enzymes are included.

Nevertheless, when considered in conjunction with the accompanying paper by Allen *et al.* (1992), the results reported here indicate that mammalian systems, such as the mouse lymphoma L5178Y Tk + / - test, might be more appropriate than bacterial tests for assessing the intrinsic mutagenic potential of caramel colours.

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