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Mutagenicity of five food additives in Ames/Salmonella/microsome test

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Abstract - The mutagenic activity of five food additives ($K_2S_2O_5$: potassium metabisulphite, KMB; K_2SO_4: potassium sulphate, KS; Na_2SO_3: sodium sulphite, SS; KNO_3: potassium nitrate, KN; $NaNO_3$: sodium nitrate, SN) were investigated using histidin auxotrophs TA98 and TA100 strains of *Salmonella typhimurium* in the presence or absence of S9 mix. The test substances were investigated for their mutagenic effects at non toxic concentrations of 0.83, 1.66, 3.33 and 5.00 mg/plate with and without S9 mix. All the test substances were not mutagenic on TA98 and TA100 strains of *Salmonella typhimurium* in the presence or absence of S9 mix except KS and SN. KS and SN showed a weak mutagenic effect on TA100 strain in the absence of S9 mix.

Key words: Ames test, food additives, nitrate, nitrite, sulphate, sulphite.

INTRODUCTION

Food additives are used as sweeteners, antioxidants, antimicrobial substances and artificial colours. Currently there are over 3000 additives used in processing that have a variety of functions. Preservatives are used as antimicrobial substances that retard product spoilage caused by mould, air, bacteria, fungi or yeast. These substances in foods can affect individuals who are sensitive with some type of allergy, asthma, hay fever, etc. Sulphites and nitrates are the most useful substances in food as additives. Sulphites are used primarily as antioxidant (Nair and Elmore, 2003) to prevent or reduce discoloration of light-coloured fruits and vegetables, such as dried apples and dehydrated potatoes. They are also used in wine making, for bleaching food starches, and used in the production of cellophane for packaging. Sulphites are also used in fruit juices, concentrated soft drinks, dried fruit, wine, beer, some sauces, pickles, and hamburger patties. Sulphites are listed on food labels are sulphur dioxide, sodium sulphite, sodium, and potassium bisulphite, sodium and potassium metabisulfite. In addition, nitrates and nitrites are labelled as sodium and potassium nitrate, sodium and potassium nitrite that are used in meat and for flavouring and fixing colour in a number of red meat, poultry, and fish products (National Toxicology Programme, 2001). Sulphites and nitrates have been reported to cause anaphylaxis, asthma, urticaria/angioedema, seizure, nausea, abdominal pain, diarrhoea and death (Abrams, 1983; Yang and Purchase, 1985; Yang *et al*., 1986; Belchi-Hernandez *et al*., 1993; Hawkins and Katelaris, 2000). It was also reported that some of sulphites, nitrates and nitrites are genotoxic or carcinogenic at different test systems (MacRae and Stich, 1979; Renner and Wever, 1983; Popescu and DiPaolo, 1988; National Toxicology Programme, 2001; Rencüzoğulları *et al*., 2001; Nair and Elmore, 2003).

The Ministry of Agricultural of Turkey (1997) suggested that sulphates, sulphites and nitrates might be used at a maximum dose of 300 mg/l in food as antimicrobial, antioxidant and reducing dicolourable substances.

At the present, there is no published data on the mutagenicity of sulphites, sulphates and nitrates in Ames/Salmonella/microsome test. For this reason, the aim of this study was to investigate the mutagenic effects of five food additives: potassium metabisulphite (KMB), potassium sulphate (KS), sodium sulphite (SS), potassium nitrate (KN) and sodium nitrate (SN) in Ames/Salmonella/Microsome test system.

MATERIAL AND METHODS

Chemicals. All the test substances (KMB: E224, KS: E515, SS: E221, KN: E252, SN: E251) were purchased from Merck. The purity of KMB, KS, SS, KN and SN are 99.9, 99.0, 95.0, 99.0 and 97.0%, respectively. Mutagenicity test tablets containing nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate were purchased from Boehringer Mannheim Biochemicals (Germany). DMSO (D8418), l-histidin (H8125), d-biotin (B4501), ampicilin (A6140), sodium azide (SA, S2002) and 2-aminofluorene (2-AF, A9031) were purchased from Sigma; 4-nitro-o-phenylenediamine (NPD) was purchased from Aldrich; agar (7178) was purchased from Aquamedia; nutrient broth (NB, B241116) was purchased from Oxoid; 3-methylcolanthrene (200-276-4) was purchased from Oekanal.

The recommended maximum test concentrations for soluble non-cytotoxic substances are 5 mg/plate (Redbook,

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2000). Because of that, all the test substances were dissolved in bidistilled water at the concentrations of 0.83, 1.66, 3.33 and 5.00 mg/plate. In this study, 2-AF (dissolved in DMSO), NPD (dissolved in DMSO) and SA (dissolved in distilled water) were used as positive controls.

The pH of the test substances was measured using MTW pH 315i (Weilheim) pHmeter.

Preparation of S9. The albino male rats (*Rattus norvegicus* var. *albinos*) weighting 200 g were pre-treated with 80 mg/kg concentration of 3-methylcolanthrene (dissolved in sunflower oil) for 5 days and the S9 fraction and S9 mix were prepared following the procedure of Maron and Ames (1983). S9 fraction (microsome fraction) was prepared and immediately distributed to small plastic tubes at 1 ml quantity and then stored at –35 ºC. The S9 mix was prepared fresh for each mutagenicity assay. For preparation of S9 mix, one S9 tablet dissolved in 18 ml of sterile bidisitilled water supplemented with 2 ml of microsome fraction; 0.5 ml of S9 mix was used for each plate. S9 mix contains microsome fraction, NADP, G-6-P and other salt solutions (Maron and Ames, 1983).

Bacterial strains. Histidin deficient (his^-) tester strains TA98 and TA100 of *Salmonella typhimurium* were kindly provided by J.L. Swezey, Curator, ARS Patent Culture Collection, Microbial Genomics and Bioprocessing Research Unit, North University Street, Peoria, Illinois 61604, USA. The TA98 strain is used for the detection of frameshift mutagens and TA100 strain for the detection of base pair substitution mutagens. Before use in the assay, each strain was checked for the presence of strain-specific marker as described by Maron and Ames (1983).

Mutagenicity assay. The standard plate-incorporation assay was examined with *Salmonella typhimurium* TA98 and TA100 in the presence or absence of S9 mix according to Maron and Ames (1983). 0.5 ml of S9 mix containing per plate was used for the assay. For the test mutagenicity, all the test substances were dissolved in distilled water, autoclave sterilized (121 °C, 20 min) and used as 0.83, 1.66, 3.33 and 5.00 mg concentrations per plate. Toxicity of these chemicals to bacteria was observed based on the significantly reduced number of revertants compared to spontaneous control. On the other hand, 4-nitro-o-phenylenediamine and sodium azide were used as positive mutagens for TA98 and TA100, respectively. In the presence of S9 mix, 2-aminofluorene was used as positive mutagen for both TA98 and TA100 strains. Each sample was examined with a three plate count and all experiments were performed twice.

Statistical significance. The significance between control revertants and revertants of treated groups were determined using t-test. Dose-response relationships were determined using regression and correlation (r) test systems.

TABLE 1 – The mutagenicity of potassium metabisulfite, potassium sulfate, sodium sulfite, potassium nitrate and sodium nitrate in *Salmonella typhimurium* TA98 and TA100 strains in the absence (–S9) or presence (+S9) of S9 mix

Test substances	Concentration (mg/plate)	TA98		TA100	
		– S9	+ S9	– S9	+ S9
Spontaneous control	–	13.00 ± 2.89	12.60 ± 1.50	104.75 ± 6.80	122.00 ± 10.7
NPD	30 µg/plate	1740.5 ± 191.13	–	–	–
2-AF	30 µg/plate	–	2204.67 ± 226.73	–	2723.30 ± 167.20
SA	2 µg/plate	–	–	981.33 ± 52.68	–
$K_2S_2O_5$ (KMB)	0.83	10.83 ± 1.49	11.00 ± 1.18	111.00 ± 5.40	108.30 ± 2.40
	1.66	7.67 ± 0.80	12.00 ± 1.79	117.83 ± 5.56*	110.00 ± 9.50
	3.33	13.33 ± 1.82	12.80 ± 1.77	104.75 ± 6.41	110.00 ± 9.70
	5.00	12.50 ± 1.15	13.20 ± 1.59	116.60 ± 16.2	118.00 ± 4.00
K_2SO_4 (KS)	0.83	10.50 ± 2.86	15.80 ± 0.92*	125.87 ± 11.5	132.70 ± 4.30
	1.66	12.67 ± 1.45	11.80 ± 1.28	136.29 ± 8.30*	116.00 ± 16.1
	3.33	11.33 ± 2.04	15.20 ± 1.16*	144.75 ± 9.98*	121.50 ± 5.30
	5.00	13.67 ± 1.63	13.40 ± 0.93	138.40 ± 4.30*	117.80 ± 7.60
Na_2SO_3 (SS)	0.83	8.83 ± 1.78	15.40 ± 1.60	148.80 ± 20.9	103.70 ± 3.90
	1.66	9.67 ± 1.41	12.40 ± 1.21	145.83 ± 15.8*	105.00 ± 7.40
	3.33	9.00 ± 1.46	14.00 ± 1.64	125.24 ± 9.07	97.00 ± 9.00
	5.00	9.83 ± 2.64	16.80 ± 0.97*	100.75 ± 6.98	121.00 ± 4.90
KNO_3 (KN)	0.83	12.33 ± 0.71	12.40 ± 1.57	120.80 ± 9.97	148.70 ± 19.0
	1.66	12.00 ± 1.03	14.60 ± 2.18	135.50 ± 7.53**	108.00 ± 8.30
	3.33	11.83 ± 0.75	16.20 ± 2.18	131.33 ± 14.2	103.30 ± 5.00
	5.00	12.50 ± 1.02	16.20 ± 2.44	124.67 ± 8.56	101.80 ± 2.30
$NaNO_3$ (SN)	0.83	11.40 ± 1.08	19.00 ± 1.52*	123.00 ± 6.24*	108.50 ± 5.00
	1.66	9.00 ± 1.00	15.40 ± 1.96	131.33 ± 10.4*	120.00 ± 4.60
	3.33	10.17 ± 0.48	14.60 ± 1.81	134.20 ± 9.63*	137.80 ± 8.70
	5.00	10.83 ± 1.25	12.60 ± 1.50	132.29 ± 6.59**	125.30 ± 6.90

NPD: 4-nitro-o-phenylenediamine, 2AF: 2-aminoflourene, SA: sodium azide, $K_2S_2O_5$: potassium metabisulfite, K_2SO_4: potassium sulfate, Na_2SO_3: sodium sulfite, KNO_3: potassium nitrate, $NaNO_3$: sodium nitrate. *: P<0,05; **: P<0,01.

RESULTS

Mutagenicity of five food additives was investigated by Ames/Salmonella/microsome mutagenicity test system by using the most sensitive *Salmonella* tester strains TA98 and TA100. This test was performed in both the absence and the presence of the rat liver metabolising system (S9 mix), providing a very sensitive study of potential mutagenic pathways for the metabolism of the test substances. The doses of the test compounds were tested and compared with the results of spontaneous control. Positive results were represented as an increase in the numbers of revertant colonies. In each experiment set, a suitable known mutagen was tested as a positive control and the results are presented in (Table 1 and Fig. 1).

KMB, SS and KN were not mutagenic for both TA98 and TA100 strains of *S. typhimurium* in the absence of S9 mix. On the other hand, KS and SN were not mutagenic for TA98 strain while they were mutagenic for TA100 strain in the absence of S9 mix without a dose-dependent manner (Table 1 and Fig. 1).

In the presence of S9 mix, all the test substances did not increased the number of revertant colonies when compared with spontaneous control (Table 1).

The pH of the maximum dose (5 mg/plate) of the test substances was measured in 18 ºC. The pH is 5.20 for KMB, 6.10 for KS, 8.66 for SS, 7.14 for KN and 6.01 for SN.

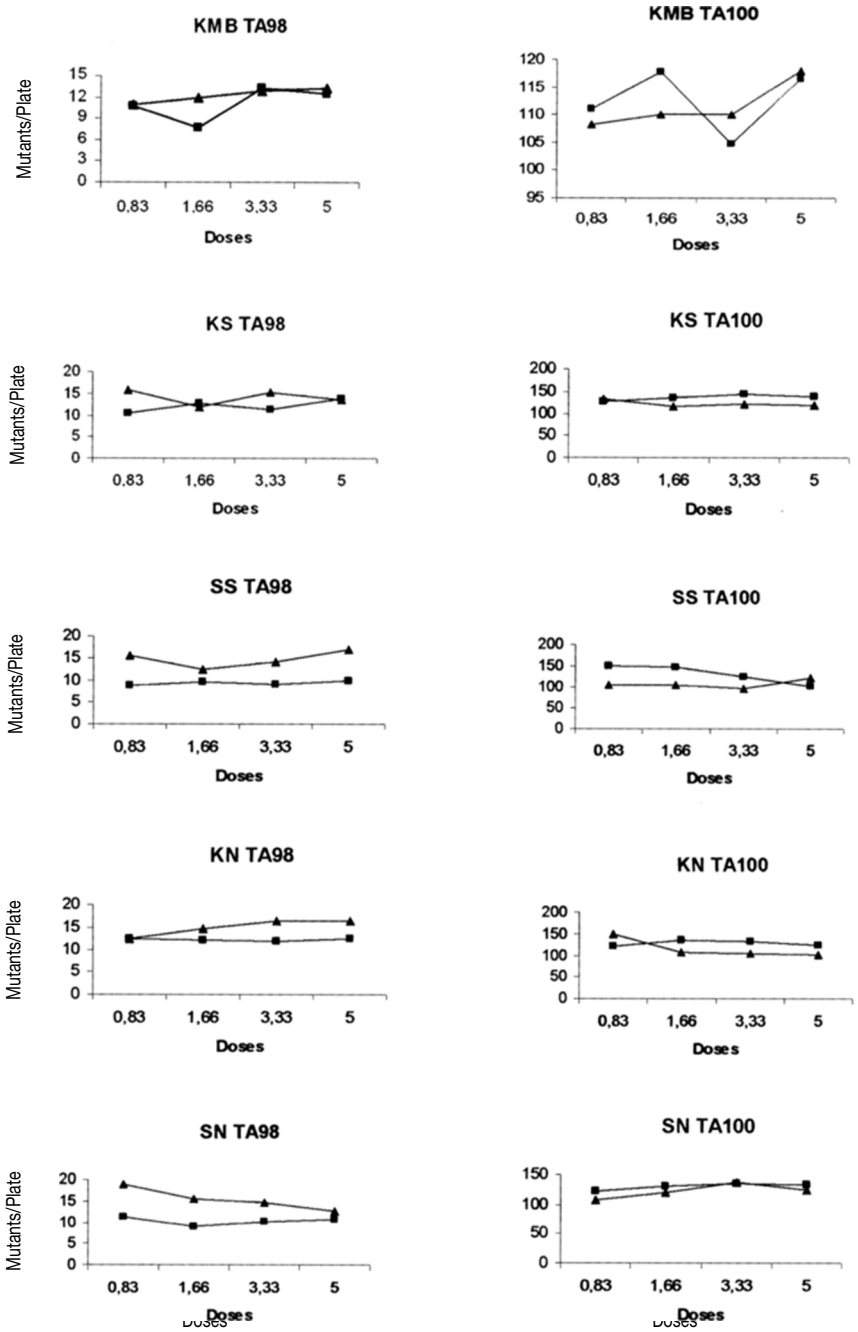

FIG. 1 – The number of the revertants induced by KMB, KS, SS, KN and SN in Ames/Salmonella/microsome test using TA98 and TA100 in the presence (▲) or absence (■) of S9 mix.

DISCUSSION

Sulphite and sulphate convert to H_2S and SO_2. Sulphites also convert to the bisulphite when dissolved in water. Bisulphite (HSO_3) causes the deamination of cytosine in both double-stranded and single-stranded DNA and in RNA (Bhanot and Chambers, 1977; Bhanot *et al*., 1977). Pagano and Zeiger (1987) reported that, the deamination of cytosine caused to base-pair substitution mutations and they also reported that sodium bisulphite was a weak mutagen at pH 5-6 in TA100 and TA97, but it was not mutagenic in TA94 and TA1537. In addition, sodium bisulphite was mutagenic in TA100 strain treated with 1 M sodium bisulphite (pH 5.2) for various time intervals (De Giovanni-Donnelly, 1985). In the present study, sulphites (KMB and SS) were not mutagenic for TA98 and TA100 in the presence or absence of S9 mix. However, sulphate (KS) was mutagenic for TA100 in the absence of S9 mix without a dose-dependent manner. The mutagenicity of bisulphite may be occurred by sodium ions (Ashby and Ishidate, 1986; Nowak, 1987, 1989). It was reported that KCl and NaCl exhibited an antimutagenic response in yeast (Parker and von Borstel, 1990) however, KCl and NaCl were both capable of inducing mutations when each were administered at a mortality of two different lengths of time of the logarithmic phase cells of the Saccharomyces cerevisiae (Parker and von Borstel, 1987). As shown, K and Na when were administered at mortality induced the mutations. In the present study, KMB and SS were not mutagenic whereas KS was a weak mutagenic. Nair and Elmore (2003) reported the same results that KMB was negative in the mutagenicity studies and Nair and Elmore (2003) also reported that KMB are safe as used in cosmetic formulations. Hayatsu and Shiragami (1979) reported that, 5-hydroxymethyl cytosine reacted with bisulphite and, instead of undergoing usual deamination process, gave cytosine 5-methylsulfonate as the product at pH 6-7. KS showed a weak mutagenic effect at pH 6.10. However, KMB and SS were not mutagenic at pH 5.20 and 8.66, respectively. On the other hand, sulphites and sulphates caused to chromosome aberrations, sister chromatid exchanges and micronucleus formations in various test systems (Njagi and Gopalan, 1982; Meng and Zhang, 1992; Rencüzoğulları *et al*., 2001).

Nitrate converts to N_2 or NH_3, nitrates also act as donor of nitric oxide (NO) that recognized a potential for genotoxicity (Andreassi *et al*., 2001). Hughes *et al*. (2000) reported that products of colonic protein degradation and metabolism include ammonia (NH_3), phenols, indoles and amines showed toxic effects in vitro and in animal models and induced the incidence of colorectal cancer. The NO was used to nitrosation of the compounds *in vivo* (Ohsawa *et al*., 2003; Ozhan and Alpertunga, 2003). It was reported that, the nitroso compounds were the most genotoxic and mutagenic effects than the non nitrosoated forms (Cid *et al*., 1990; Shi *et al*., 1991; Topaktaş *et al*., 1996; Kleinsasser *et al*., 2001; Yoon *et al*., 2001; Ohsawa *et al*., 2003). Andreassi *et al*. (2001) reported that a possible genotoxic activity was observed after nitrate therapy in humans. In addition, nitrates induced the chromosome aberrations in peripheral blood lymphocytes of 70 children (Tsezou *et al*., 1996), showed a cytotoxic effect in *Allium cepa* root tip cells (Panda *et al*., 2001) and in human lymphocytes (Andreassi *et al*., 2001). However, nitrates did not induce the chromosome aberration (Andreassi *et al*., 2001) and sister chromatid exchanges (Tsezou *et al*., 1996) in humans. In the present study, KN was not mutagenic for both TA98 and TA100 strains in the presence or absence of S9 mix. However, SN showed a weak mutagenic effect for TA100 strains in the absence of S9 mix without a dose dependent manner.

As shown, there are many reports on mutagenicity and genotoxicity of sulphites, sulphates and nitrates. In addition, nitrates convert to the carcinogenic ammonia and used for nitrosation of the organic compounds that they have the most mutagenic effects in vivo system. In the present study, all the additives tested do not show any significant mutagenicity, lacking a correlation between dose and effect. KS and SN showed weak mutagenic effects. However, sulphites, sulphates and nitrates are genotoxic in the other short-term genotoxicity tests. For this reason, it is necessary to be careful when using these substances in food and cosmetics as additives.

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