THE PRODUCTION OF MUTAGENS IN THE INTESTINE OF MICE FED ON A DIET CONTAINING 15 % SORBIC ACID (I)

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Accepted January 18, 1983

Abstract.....When mice were fed on a diet containing 15 % sorbic acid for a period up to 6 months, ether extracts of the intestinal contents of the mice were not mutagenic with *Salmonella typhimurium* TA 98, but the acidic components obtained by fractionating the ether extracts, showed slightly mutagenic activity with *S. typhimurium* TA 98, and they required the addition of liver 9,000 xg supernatant fraction for mutagenic activation.

These results suggested that mutagens were gradually produced in the intestine and moved into the liver where they were metabolically activated.

It is possible to presume that liver carcinoma might be attributable to the production of these mutagens.

Key words : Production of mutagens, mutagens in the intestine, sorbic acid, 15 % sorbic acid diet, intestinal mutagens.

INTRODUCTION

Sorbic acid (SA) and potassium sorbate (SA-K) are very widely used as antimicrobial preservatives in the food industry, and they have demonstrated very low acute or chronic toxicity for experimental animals (FDA report, 1975).

Tests of SA and SA-K for mutagenicity in *S. typhimurium* TA 92, TA 1535, TA 100, TA 1537, TA 94 and TA 98 were negative (Ishidate Jr. *et al.*, 1980).

However, a carcinogenic effect on the liver has been demonstrated in mice given dietary levels up to 15 % for 88 weeks.

Ishizawa *et al.* (1980) investigated the mutagenicity of the tissue samples of mice which were fed on a diet containing 15 % SA for 88 weeks, and no samples were found to be positive in the *B. subtilis* rec-assay.

Recently, the mutagenic activity of SA in the presence of nitrite in an acidic environment was reported by Kada *et al.* (1974). One of the known highly mutagenic

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compounds obtained under these conditions has been identified as ethyl nitrolic acid (Namiki *et al.*, 1975).

The metabolism of 1-¹⁴C-SA has been studied in respect of mice by Westöö (1964); 81%±10% of SA was oxidized to CO₂, the dose given ranging from 40-3000 mg/kg b. w. About 7% of the activity was excreted in urine as SA and 0.4% as *trans, trans*-muconic acid.

Elucidating the cause of the induction of hepato-carcinoma reported by Ishizawa *et al.* (1980), the authors have presumed the production of certain mutagens in the intestine, which were activated by liver enzymes during a long-term high-level dose of SA.

We show here that the intestinal contents of mice have been mutagenic in respect of *S. typhimurium* TA 98 under liver 9,000xg supernatant fraction (S9) mix during a long-term dose of 15% SA.

EXPERIMENTAL

Chemicals Sorbic acid (SA) and potassium sorbate (SA-K), food additives, were purchased from Takeda Yakuhin Kogyo, Osaka, 2-aminoanthracene and furyl furamide (AF-2) were gifts of Dr. Yoshikawa, National Institute of Hygienic Sciences, Tokyo. Nutrient broth was purchased from Difco Laboratory, Detroit, Michigan, U. S. A., the hepatic 9,000xg supernatant fraction (S9) of Sprague-Dawley rat treated with phenobarbital and 5, 6-benzoflavone was from Oriental Yeast Co. Ltd., and NADPH, NADH and G-6-P were from Oriental Yeast Co. Ltd.

Tester strains

The *Salmonella typhimurium* tester strains TA 98 and TA 100 were kindly supplied by Dr. Kunie Yoshikawa, National Institute of Hygienic Sciences, Tokyo.

The genetic markers of these strains (histidine requirement, deep rough character, ampicillin resistance) were routinely checked as described by Ames *et al.* (1975).

Animals and treatments

Four-week old Jcl/ICR male mice were obtained from Nihon Kurea Co. Japan.

Animals were housed in an air-conditioned room maintained at 25±1°C and were given Nihon Kurea Co. Animal Diet CE-2 and water *ad lib*.

Feeding study

Groups of 77-79 male mice were housed five or six in a cage and fed on diets containing 0 (control) (1G), 1.34%-(2G), 6.7%-(3G), 20.1%-SA-K (5G) or 15%-SA (4G) for 6 months. The composition of the diet was as listed in Table 1. The mice were weighed at approximately weekly or monthly intervals up to 1 yr.

Preparation of samples for the mutation assay.

1) Intestinal contents

At 1 week, 1, 3 and 6 months, intestinal contents (small and large intestinal contents) were removed from 5 to 10 mice of all groups (1-5G), and stocked at -20°C until use.

2) Extraction procedures

Intestinal contents of 5 (3 months) or 10 mice (1 week, 1 and 6 months) were put

Table 1. Composition of the Diet

Materials*	For the mice (4G, 5G) fed diet	For the mice (1G, 2G, 3G) fed diet
SA-K	20.1 %(5G)	1.34%(2G) 6.70%(3G)
SA	15.0 %(4G)	
Hokuyō meal	13.5 %	10.0 %
Wheat flour	17.3 %	27.3 %
Corn	10.0 %	18.0 %

* : Vitamins and inorganic ingredients contained in the diet for the mice (1-5G) were similar in concentrations to those of a CE 2, basal animal diet.

together and homogenized by stirring with glass sticks and tested whether their pHs were near 7.0 as shown by the pH indicating BTB paper, then extracted four times with excess diethylether (in total, 8 volumes). The ether layer was evaporated by standing at room temperature and dried over P_2O_5 in a vacuum. The deep brown materials obtained was weighed. One part was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 70 mg/ml for the mutagenicity assay test, and the other part was subjected to fractionation (basic-, neutral- and acidic- components), using the following procedure.

The ether extracts were diluted with 0.01 N HCl (10 mg/ml of 0.01 N HCl) and extracted 3 times with CH_2Cl_2 to obtain the acidic and neutral components in CH_2Cl_2 . The pH of the aqueous phase was adjusted to pH 12 with NaOH and the aqueous phase again extracted with CH_2Cl_2 to obtain the basic components. The acidic components were recovered from the CH_2Cl_2 extract containing acidic and neutral components by extracting with 0.01 N NaOH. The aqueous phase was then acidified to pH 2 with HCl and again extracted with CH_2Cl_2 to obtain the acidic components in CH_2Cl_2 solution. Each of the three CH_2Cl_2 extracts containing the acidic, neutral and basic components was then concentrated to near dryness on a rotary evaporator and dried over P_2O_5 in a vacuum. The residue was dissolved in DMSO for the mutagenicity assay test.

Mutation assay

The assay was carried out as described by Ames *et al.* (1975) with some modifications (Yahagi *et al.*, 1977). To a tube containing the test sample (usually 0.14-3.5 mg/tube) was added 0.5 ml of S9 mix, 0.5 ml of 100 mM phosphate buffer (pH 7.4), and then 0.1 ml of the cell suspension of bacterial tester strain. The mixture pre-incubated at 37°C for 20 min was mixed with 2 ml of melted top agar at 45°C containing 0.1 μ mole of D-biotin and 0.1 μ mole of L histidine, and poured onto a minimal-glucose agar plate. After incubation at 37°C for 2 days, the revertant colonies were counted.

Duplicate plates were prepared for obtaining one datum point, and the average values are presented in this paper. The number of revertant colonies on experimental plates varied less than 15% from assay to assay. Each assay had minimum spontaneous

revertants of TA 98 and TA 100 without the S9 mix amounting were 28 ± 5 and 120 ± 20 , respectively. The numbers of spontaneous revertants were 40 ± 6 for TA 98 and 125 ± 15 for TA 100 when the mammalian enzyme system was included in the assay system.

It was confirmed in every case that His⁺ revertant colonies on the plates were observable on the surface of a lawn of unmutated bacteria.

Results are expressed as mutagenic ratios by the use of the expression of Ehrlich *et al.* (1979). The mutagenic ratio is the number of His⁺ revertant colonies on the test plates, in which the sample was run, divided by the number of His⁺ spontaneous revertant colonies on control plates. Therefore, the higher the mutagenic ratio, the more mutagenic the sample. It was generally considered that the samples were positive for the presence of mutagens if the mutagenic ratio was equal to or greater than 2.0.

RESULTS AND DISCUSSION

Table 2 indicates the ether extract yield from the intestinal contents over a period of 6 months. The average of the ether extracts was about 2% and no difference in the average was observed between the control group (1G) and the 15% SA group (4G).

Table 2. Body weight, intestinal contents weight and the recovery of the ether extracts in the five groups over a period of 6 months.

	1G	2G	3G	4G	5G
Body weight (g)*					
1 week	32.9	31.9	31.1	32.1	26.3
1 month	38.8	37.3	37.0	38.0	31.0
3 months	42.9	43.0	38.6	41.1	35.2
6 months	42.7	43.8	40.0	42.4	31.3
Intestinal contents (g)*					
1 week	1.35	1.37	1.57	1.45	1.56
1 month	1.50	1.19	1.56	1.83	2.11
3 months	1.51	1.75	1.53	2.13	1.45
6 months	1.45	1.42	1.64	1.83	1.48
Recovery of ether extracts (%)*					
1 week	1.9	2.0	2.1	2.1	1.9
1 month	2.0	2.1	2.0	2.7**	2.0
3 months	2.2	2.3	1.7	1.8	2.2
6 months	2.1	1.6	1.4	1.4	1.4

*; Data are mean.

**; The tube accidentally broke, and the sample ran out.

Ten mice for 1 week, 1 month and 6 months or 5 mice for 3 months were used in these experiments of each group.

Figure 1 shows the mutagenic ratios obtained for ether extracts with *S. typhimurium* TA 100. None of the samples of the five groups (1-5G) were positive with *S. typhimurium* TA 100. Further experiments with *S. typhimurium* TA 100 were unsuccessful, because extensive killing of bacteria took place in the presence of ether extracts of intestinal contents as reported by Hayatsu *et al.* (1981).

Extracts of mutagen acting on *S. typhimurium* TA 98 were not detected in these groups (1-5G) (Fig. 2).

Fig. 1. Mutagenic ratio of ether extracts prepared from intestinal contents of the mice fed on a SA or SA-K diet for 1 week, 1 month or 3 months in respect of TA 100.

Mutagenic ratio was calculated as described in "Experimental", and *S. typhimurium* TA 100 was used in this experiments. Five (3 months) or ten mice (1 week and 1 month) per one group were used in these experiments, having been fed on a control (○, 1G), 1.34 % SA-K (●, 2G), 6.70 % SA-K (▲, 3G), 15.0 % SA (×, 4G) or 20.1 % SA-K (■, 5G) diet, respectively, for 1 week (a), 1 month (b) or 3 months (c).

Fig. 2. Mutagenic ratio of ether extracts prepared from intestinal contents of the mice fed on a SA or SA-K diet for 1 week, 1 month, 3 months or 6 months in respect of TA 98.

S. typhimurium TA 98 was used in this experiment. Mice were used which had been fed under similar conditions to those given in Fig. 1.

Hayatsu *et al.* (1981) indicated that an ether extract of normal human feces exhibits inhibitory effects on the activities of several mutagens in the Ames tests.

In an attempt to assay the mutagens after the separation from inhibitory compounds in the biological samples, experiments with *S. typhimurium* TA 98 were carried out on acidic, neutral and basic components obtained by fractionating the ether extracts (Fig. 3-5).

Neither basic nor neutral components of the two groups (1 and 4G) were mutagenic (Fig. 3 and 4), but acidic components of 15% SA-group (4G) showed slightly mutagenic activity at 3 and 6 months (Fig. 5), to *S. typhimurium* TA 98 when S9 was present. It seemed that mutagens co-existed in the acidic components. Mutagenic ratios of acidic components at 6 months were almost as high as those at 3 months, and the distribution of the acidic components at 6 months was 1.5 times higher than that at 3 months (Table 3). These results suggested that mutagens were gradually produced in the acidic components of ether extracts during 6 months.

Mutagens in intestine of mice fed on a sorbic acid diet

Fig. 3. Mutagenic ratio of basic components of ether extracts derived from the mice fed on a SA diet for 3 or 6 months in respect of TA 98.

Basic components were prepared as described in "Experimental". Mice of two groups (1G or 4G) were used in this experiment, which had been fed under similar conditions to those given in Fig. 1.

Fig. 4. Mutagenic ratio of neutral components of ether extracts derived from the mice fed on a SA diet for 3 or 6 months in respect of TA 98.

Neutral components were prepared as described in "Experimental". Mice of two groups (1G or 4G) were used which had been fed under similar conditions to those given in Fig. 1.

Fig. 5. Mutagenic ratio of acidic components of ether extracts derived from the mice fed on a SA diet for 3 or 6 months in respect of TA 98.

Acidic components were prepared as described in "Experimental". Mice of two groups (1G or 4G) were used which had been fed under similar conditions to those given in Fig. 1.

Table 3. Distribution of the acidic, basic and neutral components in ether extracts.

Components	Distribution in ether extracts (%)			
	Feeding periods			
	3 Months		6 Months	
	1G	4G	1G	4G
Acidic	21	23	32	36
Neutral	62	70	70	58
Basic	4.3	5.5	5.8	9.2

Hayatsu *et al.* (1981) pointed out that care must be taken in screening mutagens by the Ames tests to avoid false negatives resulting from the presence of unsaturated fatty acids such as oleic and linoleic acids in fecal extracts. In fact, these unsaturated fatty acids showed strong inhibitory activities under their experimental conditions (Hayatsu *et al.*, 1981). Mutagenicities of acidic components were tested in the presence of oleic and linoleic acids, so that they were considered to be inhibited by these acids, although our assay conditions differed in respect of the volume of S9 from theirs (Hayatsu *et al.*, 1981).

Generally, there are some problems of quantifying and scaling mutagenicity data obtained by testing complex biological mixtures such as feces. For instance, fecal extracts enhance mutagenicity (Reddy *et al.*, 1980) but fecal extracts inhibit mutagenicity (Bruce *et al.*, 1977; Hayatsu *et al.*, 1981).

Our experimental results suggest the possibility that mutagens are produced in the intestine of the mice which have been fed on a diet containing 15% SA for 6 months. Probably, these mutagens are absorbed in the intestinal wall and moved into the liver where they are activated by the S9 fractions.

Therefore, one cause of liver carcinoma is considered to be the production of these mutagens owing to the ingestion of a 15% SA diet.

It was reported by Ishidate Jr. *et al.* (1980) that SA was not mutagenic towards *S. typhimurium* TA 98, so that mutagen present in the acidic components was not SA.

As for the metabolites of SA, *trans, trans*-muconic acid and CO₂ were known in mice (Westöö, 1964), and sorbaldehyde, *trans*-4-hexenoic acid and ethyl sorbate were also known in microorganisms (Kurogochi *et al.*, 1974; Tahara *et al.*, 1977). As for the mutagenicities of the active compounds similar to these metabolites, a series of aliphatic epoxide was assayed by Wade D. R. *et al.* (1978) and Wade M. J. *et al.* (1979). As a result, the epoxides in their studies were found to be not or little mutagenic in strain TA 98. In our experiments, mutagens present in the acidic components were found to be mutagenic towards *S. typhimurium* TA 98. Therefore, it was supposed that these mutagens might be other substances except for such metabolites described above.

Further, these intestinal mutagens are not the products of the reaction between SA and nitrite, because the products were directly mutagenic in the absence of S9 mix, although the intestinal samples required the addition of S9 to the plate for activation.

Consequently, the chemical structures of mutagens are not clear at present until the purification of mutagens is successful.

Ether extracts of liver were not mutagenic with *S. typhimurium* TA 98 in our laboratory.

The variation of mutagens is being further investigated for longer periods, and the purification of mutagens is being actively carried out in our laboratory.

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