URINARY EXCRETION OF MUTAGENS AND THE EFFECTS OF SORBIC ACID ON THE LIPID PEROXIDE LEVEL IN THE MICE FED ON A 15% SORBIC ACID DIET.

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Abstract.....The urine of the mice fed on a 15% sorbic acid diet was treated with or without β -glucuronidase and was fractionated by XAD-2 column chromatography. The non-polar urine fraction was slightly mutagenic towards TA 98 when metabolically activated, but not towards TA 100.

From the comparison of thin-layer chromatograms between the intestinal and urinary samples, it was suggested that a part of the mutagens produced in the intestine was excreted in the urine.

As for the lipid peroxidation, the levels of lipid peroxide in the liver of the mice fed on a 15% sorbic acid diet were lower than those in the control over the feeding period of 15 months. Moreover, there was a correlation between the concentration of sorbic acid (X) in the diet and the lipid peroxide level (Y) in mice fed on potassium sorbate diets, obeying the linear equation, $Y = -8.39X + 341$ ($p < 0.01$). However, the lipid peroxide levels of 15% sorbic acid group did not fit with the above equation, and was higher than those of 20.1% potassium sorbate group, which was equivalent to 15% sorbic acid group in respect of sorbic acid concentration. Accordingly, the difference of lipid peroxide levels between the two groups (15% sorbic acid and 20.1% potassium sorbate group) might reflect productive difference of the mutagens.

Key words : Urinary mutagens, lipid peroxide level, sorbic acid, 15% sorbic acid diet.

INTRODUCTION

Aiso's group found the incidence of hepatoma in the mice fed on a 15% sorbic acid (SA) diet for a long period (Ishizawa *et al.*, 1980), and they further ascertained the reproducibility of the hepatoma, and also detected the high incidence of renal cystadenoma (Aiso, *et al.* 1981).

Recently, Tsuchiya and Yamaha (1983) reported that certain mutagens were

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produced in the intestine of the mice fed on a 15% sorbic acid diet, and suggested that these mutagens might be transferred to the liver and metabolically activated.

Accordingly, it was expected that these mutagens overflowed from the organ and excreted in the urine. In the present paper, therefore, the mutagenicity of the urinary samples was assayed by using two Ames tester strains such as TA 98 and 100, though the latter was not appropriate to use as a tester strain, because of killing action of the intestinal samples (Tsuchiya *et al.* 1983).

On the other hand, it was reported that the subacute administration of SA to rats induced fat deposition in the internal organs (WHO Food Additive Series, 1974)) and that 70% of the catalase activity was inhibited by 0.112% SA *in vitro* (Lück, 1957).

These observations prompted us to speculate a possibility that formation of lipid peroxides might occur in the hepatic cells by alternation in the antioxidant balance, just as seen in the liver injury induced by other types of hepato-toxic chemicals.

The role of lipid peroxidation in carcinogenesis yet remained obscure in spite of extensive studies by many investigators (Goto, 1981).

Then, the present paper also reports on the lipid peroxide levels of liver of mice fed at various concentrations of SA and potassium sorbate (SA-K).

EXPERIMENTAL

Chemicals

SA and SA-K were purchased from Takeda Yakuhin Kogyo, Osaka, 2-aminoanthracene and furyl furamide (AF-2) were gifts of Dr. Kunie Yoshikawa, National Institute of Hygienic Sciences, Tokyo. Nutrient broth was purchased from Difco Laboratories, Detroit, Michigan, U. S. A., the hepatic 9000×g supernatant fraction (S9) of Sprague-Dawley rat treated with phenobarbital and 5, 6-benzoflavone, NADPH, NADH and G-6-P were from Oriental Yeast Co. Ltd., 2-thiobarbituric acid (TBA) and 1, 1, 3, 3-tetraethoxypropane (TEP) were from Wako Pure Chemical Industries Ltd., and β -glucuronidase (GL) (*E. coli* Type VII) was from Sigma Chemical Co.. All other chemicals used were of the highest available quality.

Tester strains

The *Salmonella typhimurium* tester strains TA98 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, and fed under the similar conditions as described previously (Tsuchiya *et al.* 1983).

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 12 months. Compositions of the diet were described previously (Tsuchiya *et al.*

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1983). The mice were weighed at approximately weekly or monthly intervals up to 15 months.

Urine collection

Male Jcl/ICR mice (Nihon Kurea Co.) were housed in individual stainless steel metabolic cages in groups of up to 5 mice per dose level per cage. Urine was collected in glass bottles surrounded by dry ice. This urine was thawed to measure volume, then stored at -20°C .

GL treatment

Urine was thawed, adjusted to pH 7.0 with 0.1 N NaOH or 0.1 N HCl solution, and centrifuged to remove sediments. Equal volumes of bacterial GL solution (130 units/ml in 0.1 M $\text{NaH}_2\text{PO}_4\text{--Na}_2\text{HPO}_4$, pH 7.0) and urine were mixed, filter-sterilized, and incubated at 37°C for 24 hr by the modified procedure of Durston *et al.* (1974).

Column chromatography of urine with XAD-2

Glass columns of $14.5\text{ cm} \times 3.5\text{ cm}$ were packed with 18 g of purified XAD-2 resin and rinsed with 100 ml of water. A flow speed of urine sample was adjusted to 3 ml per minute. Aliquots of urine (10–20 ml) were thawed, and applied to the XAD-2 column. The same procedure was carried out on the remainder of the urine that was incubated with GL before extraction (see below). After washing with 50 ml of water, the columns were eluted with 100 ml of acetone which was later evaporated to dryness at about 45°C in a rotary evaporator. The residue was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 50 mg/ml, and used in the Ames Test.

Mutation assay

Spot test was carried out according to the procedure described by Ames *et al.* (1975) and the Ames assay (pre-incubation method) was followed to the procedure described previously (Tsuchiya *et al.* 1983). Duplicate plates were prepared for obtaining one datum point, and the average values were 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 the numbers of spontaneous revertants were 28 ± 5 without the S9 mix and 40 ± 6 with the S9 mix.

It was always confirmed to observe His⁺ revertant colonies on the surface of a lawn of unmutated bacteria.

Results were also expressed as mutagenic ratios by the use of the expression of Ehrlich *et al.* (1979), and it was considered that the samples were positive for the presence of mutagens if the mutagenic ratio was equal to or greater than 2.0 as described previously (Tsuchiya *et al.* 1983).

Assay for lipid peroxides in liver by TBA

Lipid peroxides in liver were determined by the method of Ohkawa *et al.* (1979). TEP was used as an external standard, and the levels of lipid peroxides were expressed as nmol of malondialdehyde (MDA) per gram of liver.

RESULTS AND DISCUSSION

Urinary volumes and pHs were compared between the control and 15% SA group of the mice fed for 6 months. The volume of 24 hr urine of five mice was 3–8 ml in control group and 5–15 ml in 15% SA group. The pH of urine was about 6.3 in the control group and about 5.7 in the 15% SA group.

Comparing with the control group, the urinary volume tended to increase and the pH of urine to decrease in 15% SA group.

Urine was centrifuged at 15,000 rpm for 10 min to remove sediments, and filter-sterilized. One hundred μ l of urinary samples were assayed by spot test with TA 98 and TA 100.

All samples (1–5 G) were not mutagenic towards both strains. Therefore, the urinary samples of 3 groups (1,4 and 5G) were assayed up to 200 μ l of sample solution by the pre-incubation method, but any samples were not mutagenic towards both strains (Table 1). Then, the samples fractionated with XAD-2 column chromatography were assayed with Ames tester strain TA 98 (Fig. 1 (a)).

As a result, the samples of 15% SA group were mutagenic under S9 mix, but the treatment of GL did not enhance the mutagenic ratio.

Further, the fractionated samples were subjected to thin-layer chromatography (TLC) on silica gel (WAKO 5B, 1.5 mm thickness) with chloroform-methanol (15 : 1) as a developing solvent. Usually, 80 mg of the sample was loaded on a 20 cm wide plate. After development of the chromatogram, the three fractions (Fr. a (lower), Fr. b (middle) and Fr. c (upper)) were scratched off and eluted successively with ether, methanol and dichloromethane (Fig. 1 (b) and (c)).

With or without treatment of GL, upper fractions (Fr. c in Fig 1 (b) and (c)) of TLC indicated the highest mutagenic ratio among three fractions in the presence of S9 mix.

Urine of the mice fed on a 15% SA diet for 12 months was collected and also subjected to the XAD-2 column chromatography (Fig. 2).

Table 1 Mutagenicity of urinary samples assayed by pre-incubation method.

		Revertants per petri dish											
		TA 98						TA 100					
		– S9			+ S9			– S9			+ S9		
μ l/plate		50	100	200	50	100	200	50	100	200	50	100	200
	1G	40	78	88	35	60	56	152	152	187	160	186	239
Pre-inc.	4G	28	43	40	46	46	56	140	152	164	159	160	146
method	5G	45	45	33	39	39	42	140	140	129	173	160	159

Mutation assay was carried out according to the procedure described in "Experimental". The values given are the average of duplicate plates. Average number of spontaneous revertants: TA 98, 25 (– S9), 35 (+ S9); TA 100, 117 (– S9), 133 (+ S9).

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Fig. 1 Mutagenic ratio of urinary samples prepared with XAD-2 column chromatography in respect of TA98.

(a) Animals were fed on a 15% SA diet for 6 months and the preparation of urinary samples was described in "Experimental".

○, 1G; ●, 1G+GL; △, 4G; ▲, 4G+GL.

(b) and (c) Urinary samples ((b) without or (c) with the treatment of GL) of 15% SA group (4G) loaded on TLC were developed and three fractions were extracted as described in the "Text". The extracts were dissolved in DMSO at the concentration of 30 mg/ml.

Yield (b); Fr. a (R_f 0-0.22, △), 35%; Fr. b (R_f 0.22-0.52, □), 28%; Fr. c (R_f 0.52-1.0, ○), 37%.

Yield (c): Fr. a (R_f 0-0.28, ▲), 39%; Fr. b (R_f 0.28-0.66, ■), 24%; Fr. c (R_f 0.66-1.0, ●), 37%.

Oleic acid R_f ; 0.48 in (b), 0.61 in (c).

Fig. 2 Mutagenic ratio of urinary samples prepared with XAD-2 column chromatography.

(a) Animals were fed on a 15% SA diet for 12 months. Urinary sample was prepared and assayed as described in "Experimental". ●, TA 98; ×, TA 100.

(b) Three fractions of urinary sample of the mice fed on a 15% SA diet for 12 months were prepared as described in the "Text".

Yield: Fr. a (R_f 0-0.43, Δ), 25%; Fr. b (R_f 0.43-0.61, $\square$), 46%; Fr. c (R_f 0.61-1.0, $\circ$), 29%. Oleic acid R_f : 0.54 in (b). TA 98.

It was found that urinary samples were not mutagenic towards TA 100, but mutagenic towards TA 98 in presence of S9 mix (Fig. 2 (a)). Next, the samples were also subjected to TLC and fractionated according to the similar procedure described in Fig. 1 (b) and (c) except for no treatment of GL (Fig. 2 (b)).

As a result, the mutagenic ratio was decreased at more than 4 mg per plate owing to killing substance mostly existed in the upper fractions (the number of revertant colony was nil at the concentration of 4 mg per plate in Fr. c).

Thin-layer chromatograms of acidic components of intestinal contents and urinary samples were compared between the control and 15% SA group (Fig. 3). A few spots in the acidic components of the intestinal contents of 15% SA group were not detected in the controls. Therefore, some of these spots might be mutagens. Further, upper two spots of urinary samples of 15% SA group were also detected in the acidic components of 15% SA group. It was considered that two spots might be the mutagens that were excreted into urine after the absorption from the intestinal contents.

It was reported (WHO Food Additive Series, 1974) that the cholesterol value in blood and the lipid accumulation in internal organs were increased in the rat fed on 10% SA diet for 4 months. On the other hand, it was also known that SA showed a strong inhibitory effect on catalase at the concentration of 0.112% (Lück, 1957). Accordingly, there was a possibility that lipid peroxidation had made progress for a long period of feeding, so, the lipid peroxide level of liver was determined according to the TBA method of Ohkawa *et al.* (1979).

Fig. 3 Thin-layer chromatograms of urinary samples prepared with XAD-2 column chromatography.

Experimental conditions of TLC were as follows :

Plate ; Merck 60, thickness 0.2 mm. Solvent ; chloroform : methanol=15 : 1.

Detection ; plate was heated after the spraying with 50% sulfuric acid solution.

Sample amounts : Urinary samples (100-250 μ g) obtained from the mice (1 and 4G) fed for 6 months ; acidic components (200 μ g) prepared from the intestinal contents of the mice fed for 8 months according to the method described previously (Tsuchiya *et al.*, 1983).

As a result, the levels in SA group (4G) were always lower than those in the controls over a long period of feeding (Fig. 4).

Generally, it has been considered that peroxidation damage is involved in aging. Based upon our data, it was hypothesized that SA was active in preventing the auto-oxidation chain reactions which would lead to the formation of lipid peroxides, hydroperoxides or other complexes in the normal mice, and owing to this prevention of SA, the feeding on a 15% SA diet might be able to enhance the mice survival as observed in the experiments by Ishizawa *et al.* (1980). The results in Fig. 4 led to the conclusion that lipid peroxide level determined by TBA method did not give the information of the induction of hepato-carcinogenesis in our experiments, because the high level of lipid peroxide was generally expected in the hepatoma group (Tsujii *et al.* 1976 ; Kasagi *et al.* 1978).

As for SA-K group (2, 3, 5G) fed for 15 months, there was a linear relationship between the lipid peroxide level and SA concentration in diet ($p < 0.01$) (Fig. 5), but, that of 15% SA group (4G) was not fit with the equation in Fig. 5 and rather came close to the control at 15 months (Fig. 4).

It was considered that a part of the mutagens was overflowed into the urine as it

Fig. 4 Time course of lipid peroxide level in mice fed on a 15% SA diet. Lipid peroxide level was assayed according to the method as described in "Experimental". Each point is the mean of 3 animals. Bars, S. D.

Fig. 5 Relationship between the lipid peroxide level in liver and the concentration of SA in diet.

Mice were fed on the four sorts of diet (1-3, 5G) over a period of 15 months. Lipid peroxide levels of 3 animals were assayed according to the method described in "Experimental". The results are given as mean $\pm$ S. D. (Correlation coefficient of line: $r = -0.78$, $p < 0.01$).

was (Fig. 3), and the mutagenic ratios of urinary samples (Fig. 1 (a)) resulted in the values similar to the mutagenic ratios of acidic components prepared from the intestinal contents of the mice which had been fed on a 15% SA diet for 6 months (Tsuchiya *et al.*, 1983).

Namiki *et al.* (1980) reported that isolated products in the SA-nitrite reaction were directly mutagenic in the absence of S9 mix, and further indicated that the mutagenicity of 1, 4-dinitro-2-methyl pyrrole, one product of them, was destroyed by treatment with reductive reagents (Osawa *et al.*, 1980). In our experiments, the urinary samples required the addition of S9 to the plate for activation, therefore, urinary mutagens are not the products in the reaction between SA and nitrite.

There was a difference in the lipid peroxide level (15 months) between 15% SA group (4G) and 20.1% SA-K group (5G) (Fig. 4 and 5). The reason why the levels of 4G were higher than those of 5G was not clear, but the former was the group in which high incidence of hepatoma was reported (Ishizawa *et al.*, 1980).

Conclusively, in our experiments, estimation by TBA method did not indicate appreciable progression of lipid peroxidation in the liver of the mice fed on a 15% SA diet.

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