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# Characterization of Genotoxicity of Kojic Acid by Mutagenicity in Salmonella and Micronucleus Induction in Rodent Liver

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**Three lots of kojic acid (KA) which were produced for use as a reagent, food additive and in cosmetics were shown to be mutagenic in *S. typhimurium* TA100 with or without S9 mix, with a specific activity of around 100 revertants per mg of KA. Since there are contradictory reports on genotoxicity of KA, we examined, using HPLC, whether the mutagenicity to *S. typhimurium* is due to KA itself, or due to contaminants present in the KA samples. Although two UV absorbing fractions were separated by HPLC, mutagenicity was detected only in the major fraction and the specific mutagenic activity of KA did not change before and after HPLC separation. The material in the major peak fractions on HPLC was confirmed to be KA by NMR. Thus it was demonstrated that KA itself is mutagenic and no mutagenic contaminants were detected in the three lots of samples. Since KA is known to produce liver tumors in mice, we further examined the genotoxicity of KA in the liver of rodents. KA induced micronuclei (MN) in the regenerating liver of adult mice by its gastric intubation at 1 g per kg body weight. However, no MN were induced in young mice (3 weeks old) without partial hepatectomy. Since it was recently found that KA had no tumor-initiating activity in the liver of mice in a two-step carcinogenicity study, there is no evidence that the genotoxicity detected in the mouse liver is involved in liver carcinogenesis.**

**Key words: mutagenicity, liver micronuclei, genotoxicity, *S. typhimurium*, kojic acid.**

## Introduction

Kojic acid (KA) [5-hydroxy-2-hydroxymethyl]-4*H*-pyran-4-one; CAS No. 501-30-4; (Fig. 1)] is a natural substance produced by various fungi, *Penicillium* spp., *Aspergillus orizae, flavus and lamarii*, and also by certain bacteria (1). KA which had been used as a food additive for the prevention of enzymatic browning of shell fish, raw crabs and shrimp, owing to its inhibitory activity on tyrosinase, was found to be genotoxic *in vitro*, inducing *his*$^+$ reverse mutations in *S. typhimurium* (2-5). It was also found to be genotoxic *in vivo*, inducing micronuclei (MN) in peripheral blood of rats (6).

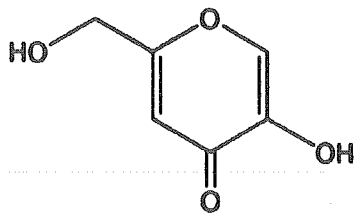


Fig. 1. Structure of kojic acid (KA).

KA had been reported to induce hepatomas and thyroid adenomas in mice (7). Thyroid adenomas or hyperplasia production in the mouse or rat was suggested to be due to promoting activity of KA (7-9). In contrast, the possibility of involvement of genotoxicity of KA in mouse hepatoma development could not be excluded (10). In 2003, the Ministry of Health, Labour and Welfare, Japan noticed that KA was no longer used for prevention of browning of shell fish in market due to the development of modern technology, and KA was withdrawn from the list of existing food additives in the same year. Since KA is a fungal product, soy sauce, miso and sake were suspected to contain KA. Out of 32 samples of fermented foods examined for the presence of KA, three samples were found to be positive, but the levels were not so high, being at the maximum, 1 ppm (personal communication by Dr. Tamio Maitani, National Health Sciences, Tokyo, Japan, Dec. 7, 2005). Further, KA is still used in cosmetics as a skin lightening agent (quasi-drug) at concentrations of up to 1.5% due

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to its inhibiting action on human melanocyte tyrosinase (11). Thus, the safety of KA needs to be confirmed from various points.

Recently, results of an extensive study on genotoxicity of KA have been reported, in which KA showed only weak mutagenicity without a dose-dependent response in *S. typhimurium* (12). Many contradictory or inconsistent results had been reported for the genotoxicity of KA. KA induced MN in peripheral blood, but not in the liver of young rats by a single application through gastric intubation (6). However, it did not induce MN in bone marrow of mice (12,13) by a single application through intraperitoneal injection. Furthermore, MN were not induced in human keratinocytes SVK14 nor in human hepatocellular carcinoma cells, HepG2, *in vitro* (12) but were induced in human lymphoblastoid cells, TK6 and WTK-1 (personal communication by Dr. Masamitsu Honma, National Institute of Health Sciences, Tokyo Japan, December 12, 2005). KA did not induce $HPRT^-$ mutations in Chinese hamster V79 cells or mouse L5178Y cells (12), but induced $TK^-$ mutation in TK6 ($TK^{+/-}$) and WTK-1 ($TK^{+/-}$, $P53^{-/-}$) cells (unpublished observations, Yu F Sasaki). As for chromosome aberrations and sister chromatid exchanges *in vitro*, positive results were reported in Chinese hamster cells (5).

Since there had been no standards for composition of KA in food hygiene law, while the manufacturers' specifications for cosmetic use was at least 97% pure, there was a possibility that the discordance reported for genotoxicity was due to differences in composition of KA samples used. Thus, it is important to clarify whether the mutagenicities of some KA samples were due to KA itself or contaminant included. In the present study, samples of various lots of KA produced by different companies that are used as a food additive, reagent and cosmetics ingredient were purified by HPLC. The resulting fractions were tested in order to determine whether the reported mutagenicity in *S. typhimurium* was due to KA itself or to another component present in the KA samples. This testing showed that the mutagenicity is due to KA itself and not to contaminants. As KA has been reported to be hepatocarcinogenic in mice (7), we further examined whether KA shows *in vivo* genotoxicity in rodent liver, by MN assay.

## Materials and Methods

**Chemicals:** Kojic acid, reagent grade, lot no. 052K2516 was purchased from Sigma (St. Louis), 5312 used for food additive (content is 100.6%) was supplied from Alps Pharmachemical Industry (Gifu, Japan) and 2Y181 [at least 97% pure, but more typically (by HPLC) was >99% pure] used for cosmetics was supplied from Sansho Seiyaku Co., Ltd (Fukuoka, Japan). For *in vivo* genotoxicity studies, KA for food additive provided by Alps Pharmachemical Industry was used. Cyclophospamide, diethylnitrosamine (DEN), dimethyl sulfoxide (DMSO), 4-nitroquinoline 1-oxide and trifluoroacetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan), and 1,2-dimethylhydrazine (1,2-DMH) and benzo[*a*]pyrene were from Tokyo Kasei Kogyo (Tokyo, Japan). Solutions of KA were prepared immediately before use.

**HPLC separation:** Analytical HPLC was performed under three systems. System 1; an LC-20A series from Shimadzu Co. (Kyoto, Japan) equipped with Shimadzu SPD-M20A photodiode detector. A Mightysil RP-18 GP column (5 $\mu$m particle, 3 mm i.d. $\times$50 mm; Kanto Chemical Co., Tokyo, Japan) was used at 40°C, with an isocratic eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) and a flow rate of 0.15 mL/min: System 2; a Shimadzu LC-10A series was used with a Mightysil RP-18 GP column (5 $\mu$m particle, 4.6 mm i.d. $\times$250 mm), an eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) and a flow rate of 0.7 mL/min: System 3; the same equipment and column with system 2, but the eluent used was 0.1% propionic acid-0.05N perchloric acid and a flow rate of 0.7 mL/min. Preparative HPLC was performed on an LC-8A series from Shimadzu Co., using a large volumetric flow cell in the UV detector. A Mightysil RP-18 GP column (5 $\mu$m particle, 10 mm i.d. $\times$250 mm; Kanto Chemical Co., Tokyo, Japan) and an eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) were used with a flow rate of 4.0 mL/min. After separation, each fraction was lyophilized and weighed. All fractions were subjected to mutagenicity testing.

**NMR analysis:** $^1H$ and $^{13}C$ spectra were recorded on an ECP-600 spectrometer (JEOL Ltd., Akishima, Tokyo) using DMSO-$d_6$ as a solvent.

**Mutagenicity assay:** The mutation assays were performed using *S. typhimurium* TA100 with and without S9 mix (14). The S9 purchased from Oriental Yeast Co. Ltd (Tokyo, Japan) was prepared from Crj:CD (SD), male rat treated with phenobarbital and 6-naphthoflavone. KA was dissolved in 0.1 mL of distilled water and the assay was performed by the method of preincubation (15). For the mutagenicity test, after separation of samples by HPLC, 1 mg of dried residues was applied to a plate for each of fractions 6, 8 and 9. For fraction 7, a dose-dependent response with 0.5, 1.0 and 1.5 mg/plate was examined and the specific activity was calculated based on the linear regression of the least square method. For other fractions, the residues were dissolved in 500 $\mu$L water, and 10 or 100 $\mu$L were applied to each plates. Statistic analysis was performed by multiple regression analysis.

**Animal and KA administration:** Male ddY mice of 3 and 8 weeks old, and male Fischer 344 rats of 8 weeks

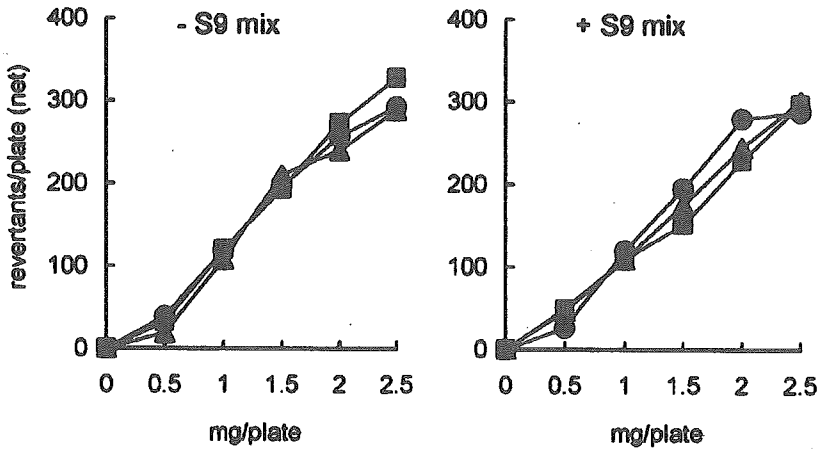


**Fig. 2.** Mutagenicities of kojic acid (KA) in *S. typhimurium* TA 100 in the absence (left) or presence (right) of S9 mix. The values were averages of two plates, subtracted with numbers of spontaneous revertants of 128 (−S9 mix) or 167 (+S9 mix). Positive control, 0.03 μg of 4-nitroquinoline 1-oxide induced 400 revertants (−S9 mix) and 5 μg of benzo[*a*]pyrene induced 1545 revertants (+S9 mix) over spontaneous one. No statistic differences were observed among these three samples. ●; lot.052K2516, ■; lot.5312, ▲; lot.2Y181.

old were purchased from SLC Japan (Shizuoka, Japan). Eight week old animals were acclimatized for one week at 24±2°C with a relative humidity of 55±5%, with basal diet of MF pellets (Oriental Yeast Industries, Tokyo, Japan) and tap water ad libitum, and were used at age 9 weeks. Three week old mice were used immediately. For gastric intubation, KA was suspended at 0, 50 and 100 mg/mL in 0.5% sodium carboxymethyl-cellulose and immediately administered to animals at a dose of 10 mL/kg, corresponding to KA doses of 0, 500 and 1000 mg/kg. The doses administered to the animals were based on the approximate maximum tolerated dose for each species determined by simple acute toxicity experiments: oral gavage with 2000 mg/kg KA resulted in death of 4/4 mice and 4/4 rats within 3h.

**Micronucleus assay:** Four mice and rats were used for each KA-dose group and three for positive control groups, following the reported method (16,17). Twenty-four hours after administration of KA, partial hepatectomy (two-thirds) was performed on 9 week old animals by removing three major lobes of the liver, the left lateral, left medial and the right lateral lobes under ether-anesthetized conditions. After four days, the animals were anesthetized with ether, and their livers were perfused *in situ* for 5 min with Liver Perfusion Medium [Hanks' balanced salt solution ($Ca^{2+}$/$Mg^{2+}$ free) containing 0.5 mM EGTA and 10 mM HEPES (pH 7.2-7.3)] (GIBCO-Invitrogen, Carlsbad, CA), followed by 5 min with Liver Digest Medium (Hanks' balanced solution ($Ca^{2+}$/$Mg^{2+}$ free) containing 0.05% collagenase type IV, 50 μg/mL trypsin inhibitor, 10 mg/mL bovine serum albumin, 10 mM HEPES, and 560 μg/mL $CaCl_2$) (GIBCO-Invitrogen). Both solutions were maintained at 37°C and delivered at a flow rate of 14 mL/min. The perfused livers were minced in a Petri dish containing Liver Digest Medium; the minced tissue was then passed through gauze, centrifuged at 200 g for 1 min, and the cell pellet obtained was fixed with 1 mL of 10% neutral formaldehyde. Ten μL of the cell suspension were mixed with 10 μL of 500 μg/mL acridine orange, the mixture placed on a glass slide and covered with a coverslip. The number of micronucleated hepatocytes (MNHEPs) among 1000 hepatocytes was recorded for each animal under a fluorescence microscope (×400 or greater), with a blue excitation filter and a yellow barrier filter. MNHEP was defined as a hepatocyte with two nuclei, one being less than 1/4 in diameter of the other nucleus. For 3 week old mice, MN assays were performed by the same procedure without partial hepatectomy, and livers were removed at 72, 96 and 120 h. The differences between the frequencies of MN in KA treated and KA 0 control animals were analyzed by one-way ANOVA followed by pairwise comparisons using the Dunnett test. A P-value of less than 0.05 was considered statistically significant.

All animal experiments were carried out following the guidelines set out by Hachinohe National College of Technology in the Guide for the Care and Use of Laboratory Animals.

## Results

**Mutagenicity in *S. typhimurium* of KA for reagent, food additive and cosmetics:** Three lots of KA, 052K2516 (reagent), 5312 (food additive) and 2Y181 (cosmetics) showed similar mutagenic activities in TA 100, under conditions without or with S9 mix (Fig. 2). All samples showed linear dose-dependent response between 0.5 and 1.5 mg/plate of KA. The specific mutagenic activities calculated by linear regression by

**Table 1.** Weight and mutagenicity in *S. typhimurium* TA100 of each fraction obtained by HPLC of KA samples

| Fr. No. | Lot. 052K2516 | | | Lot. 5312 | | | Lot. 2Y181 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Weight (mg/Fr.) | Revertants/Fr. | | Weight (mg/Fr.) | Revertants/Fr. | | Weight (mg/Fr.) | Revertants/Fr. | |
| | | −S9 mix | +S9 mix | | −S9 mix | +S9 mix | | −S9 mix | +S9 mix |
| 1 | 0.2 | 0 | 0 | 0.6 | 0 | 20 | 0.1 | 0 | 0 |
| 2 | 0.2 | 0 | 0 | 0.4 | 0 | 0 | 0.1 | 0 | 0 |
| 3 | 0.2 | 0 | 0 | 0.3 | 0 | 0 | 0.0 | 0 | 0 |
| 4 | 0.2 | 0 | 0 | 0.4 | 0 | 0 | 0.1 | 0 | 0 |
| 5 | 0.0 | 0 | 0 | 0.0 | 0 | 30 | 0.1 | 0 | 0 |
| 6 | 11.3 | 1402 | 1232 | 14.2 | 2321 | 1734 | 10.2 | 1129 | 946 |
| 7 | 22.8 | 2686 | 3051 | 27.0 | 4260 | 3504 | 33.4 | 3176 | 3377 |
| 8 | 15.1 | 1595 | 1700 | 17.4 | 2153 | 2205 | 26.6 | 2258 | 2471 |
| 9 | 7.4 | 749 | 853 | 9.0 | 945 | 1359 | 11.6 | 1170 | 961 |
| 10 | 2.9 | 292 | 213 | 2.3 | 259 | 94 | 4.1 | 410 | 250 |
| 11 | 2.0 | 0 | 0 | 0.3 | 25 | 0 | 1.2 | 0 | 0 |
| 12 | 0.8 | 0 | 0 | 0.3 | 85 | 0 | 0.2 | 0 | 0 |
| 13 | 0.4 | 0 | 0 | 0.4 | 85 | 0 | 0.2 | 0 | 0 |
| 14 | 0.4 | 0 | 0 | 0.1 | 40 | 0 | 0.2 | 0 | 0 |
| Total weight | 63.9 | | | 72.5 | | | 88.1 | | |

the least square method for 052K2516, 5312 and 2Y181 were 116, 115 and 106 revertants/mg, respectively, without S9 mix and 113, 106 and 111, respectively, with S9 mix. No statistic differences were detected among these different sources of samples at significance of $\leq 0.05$.

**Separation of mutagenic substance in KA samples by HPLC:** To clarify whether the mutagenicity was derived from KA itself or some contaminants in the samples, HPLC separations were performed. A KA sample solution in distilled water of 0.5 $\mu$g/0.5 $\mu$L was eluted by analytical HPLC under the conditions described in Materials and Methods (systems 1–3). The chromatograms under system 1 of three lots of KA monitored by absorption at 270 nm were similar, and revealed a single peak at 3.8 min (Fig. 3a). Only when a very large amount of KA was applied, a small shoulder peak was detected at the foot of the major peak (data not shown). Using two other different HPLC systems 2 and 3, similar chromatograms were obtained with three samples of KA (data not shown), with their small and very large amounts. These results indicated only a minor UV absorbing material(s) are contaminated in all three samples at similar levels.

Preparative HPLC was then applied to each lot of KA to facilitate the determination of the mutagenicity of the various constituents of the KA samples. A 500 $\mu$L aliquot of 25 mg/mL KA solution in distilled water was applied to a column, under the conditions described in Materials and Methods. Typical 270 nm chromatograms of the three samples are shown in Fig 3b. From the chromatograms, it can be seen that all of the samples include an impurity detected as a small peak shoulder, which was eluted after the major peak (arrows in Fig 3b). Circa 100 mg of each KA sample was separated by repeating the HPLC eight times, then all corresponding fractions of the eight runs were pooled and lyophilized to dryness. The contaminant distributed in fractions 10 and 11 in all three samples. The residual weight of each fraction (Table 1) correlated well with its absorption at 270 nm (Fig 3b).

When each fraction was examined for mutagenicity in *S. typhimurium* TA100, fractions 6–10 of all three samples were mutagenic with and without S9 mix, while no significant mutagenicity was detected in the other fractions, under either condition. The total mutagenicity of each fraction of the three samples (Table 1) correlated well with its absorption at 270 nm (Figs 3). All three lots of KA supplied were slightly colored, but after preparative HPLC, the purified KA (fractions 6–9) was almost colorless, and fractions 10 and 11 were strongly colored according to the original color shade.

Table 2 shows the specific activities (revertants/mg) of fractions 6–10. Since fraction 10 contained significant amounts of contaminant, the average specific activities of fractions 6–9 of each sample are also indicated, together with those of the original KA samples, calculated from the data presented in Fig 2, by linear regression by least square method. The specific activities of the three samples were almost the same before and after separation by HPLC, with and without S9 mix.

**Structure confirmation by NMR:** The dried residues of fractions 6–9 of 052K165, 5312 and 2Y181 were subjected to $^{1}H$ and $^{13}C$-NMR analysis. All of the protons in the KA structure (Fig. 1) were detected in the $^{1}H$-NMR spectra, with the following chemical shifts; 4.29 (doublet, $CH_2$), 5.65 (triplet, $CH_2$-OH, exchanges with $D_2O$), 6.33 (singlet, 5-H), 8.02 (singlet, 2-H), and

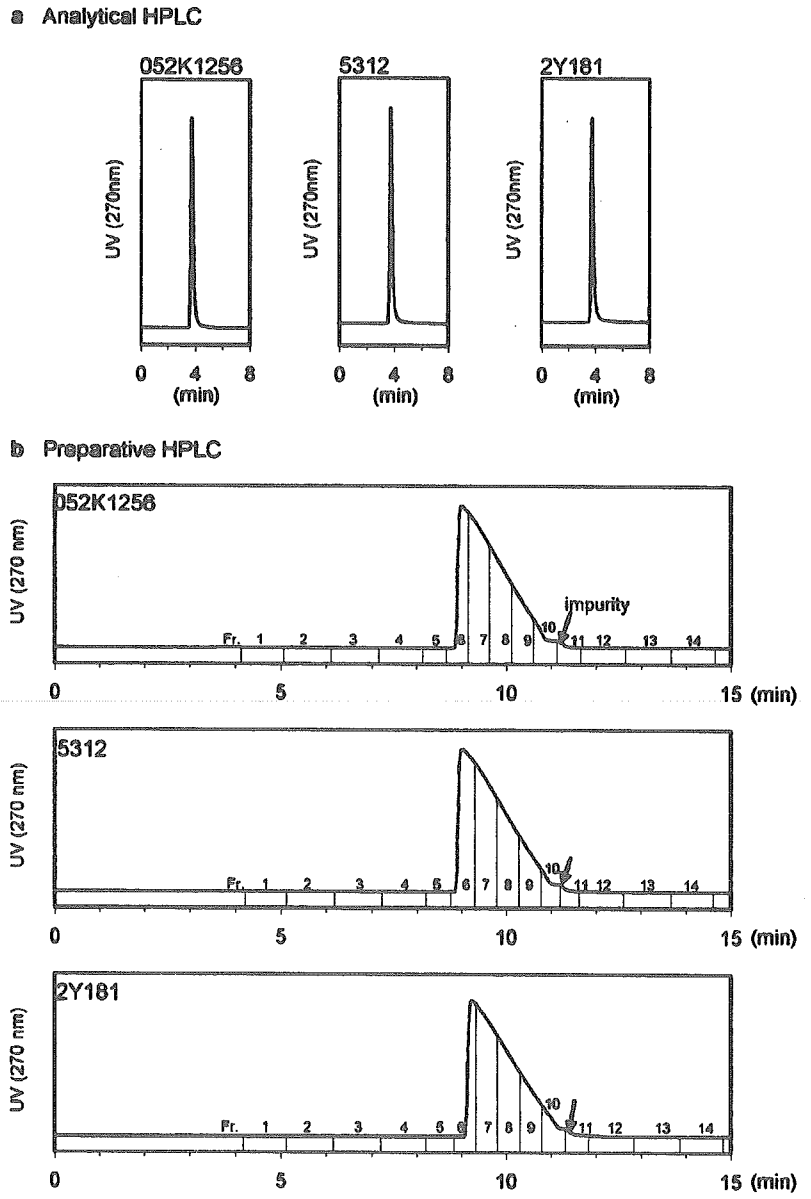


Fig. 3. Analytical (a) and preparative HPLC chromatograms (b) of three samples of KA (lot. 052K1256, 5312, 2Y181) (a): A 0.5 $\mu$L aliquot of KA solution (1 mg/mL) in distilled water was loaded on an analytical column (Mightysil RP-18 GP, 3 × 50 mm), and separated by an eluent of methanol −0.05 v/v% trifluoroacetic acid (3:97) with a flow rate of 0.15 mL/min and absorption at 270 nm was recorded. (b): A 500 $\mu$L aliquot of KA solution (25 mg/mL) was applied to a preparative column, Mightysil RP-18 GP (5 $\mu$m, 10 × 250 mm), and separated by the same eluent as that used for analytical HPLC with a flow rate of 4 mL/min. In addition to the major peak of KA, a small shoulder peak of impurity was detected as indicated by arrow.

9.05 (singlet, C=C-OH, exchanges with $D_2O$) ppm. Further, the KA carbon skeleton was detected by $^{13}C$-NMR at 60.01 (exocyclic carbon ($\underline{C}H_2$)), 110.37 (C3), 139.79 (C6), 146.25 (C5), 168.61 (C2), and 176.09 (C4(C=O)) ppm. No impurity peaks were detected in any of the samples analyzed. Thus, the major 270 nm absorbing substances in these three samples were confirmed to be KA (data not shown).

***In vivo* genotoxicity of KA:** *In vivo* genotoxicity of KA was examined by MN assay in regenerating livers of mice and rats. Four days after partial hepatectomy (120 h after KA administration), mean values of MNHPCs in mice increased dose dependently and with 1000 mg/kg of KA, the value was significantly increased as compared with 0 dose of KA (Table 3). In rats, however, no increase was observed. Furthermore, KA was found to have no MN inducing ability in infant mice without partial hepatectomy (3 weeks old) (Table 4).

Table 2. Specific mutagenicity in *S. typhimurium* TA100 of KA samples after HPLC separation

| Fraction No. | Revertants/mg | | | | | |
|---|---|---|---|---|---|---|
| | Lot. 052K1652 | | Lot. 5312 | | Lot. 2Y181 | |
| | −S9 mix | +S9 mix | −S9 mix | +S9 mix | −S9 mix | +S9 mix |
| 6 | 124 | 109 | 164 | 120 | 111 | 93 |
| 7 | 118 | 134 | 158 | 130 | 95 | 101 |
| 8 | 106 | 113 | 124 | 127 | 85 | 93 |
| 9 | 101 | 115 | 105 | 151 | 101 | 83 |
| 10 | 95 | 71 | 113 | 41 | 128 | 76 |
| Average±SEM* | 112±5.3 | 118±5.6 | 138±14 | 132±6.7 | 98±5.4 | 93±3.7 |
| Before HPLC** | 116 | 113 | 115 | 106 | 106 | 111 |

Fractions 6, 8 and 9 were analyzed with 1 mg/plate of dried substance. Fraction 7 was tested with 0.5, 1.0 and 1.5 mg/plate of the dried substance, specific mutagenicity was calculated from the linear regression by the least square method. Other fractions were tested with 2 and 20% of dried substances, and results of tests with 20% of total residue of each fraction are indicated. With 2% weight of each fraction, no more than 20% of spontaneous mutagenicity was detected. For fraction 10, the mg amount applied to a plate is; lot.052K1652, 0.58 mg; lot. 5312, 0.46 mg; lot. 2Y181, 0.64 mg. Means of duplicate assays are indicated.

*Average of specific activities of fractions 6~9±standard error of means (SEM).
**Values were calculated from Fig. 2, based on the linear regression by the least square method.

Table 3. MN induced in regenerating liver of mice and rats treated with single gavages of KA

| Species | Chemical | Dose (mg/kg) | MNHPCs/1000 HPCs (mean±SEM) |
|---|---|---|---|
| Mouse | KA | 0 | 2.33±0.33 |
| | | 500 | 5.00±1.00 |
| | | 1000 | 10.3±1.45* |
| | DEN | 160 | 15.7±1.20* |
| Rat | KA | 0 | 1.67±0.33 |
| | | 500 | 2.00±0.58 |
| | | 1000 | 1.33±0.33 |
| | DEN | 160 | 17.3±2.01* |

KA, kojic acid; DEN, diethylnitrosamine
MNHPCs, micronucleated hepatocytes; HPCs, hepatocytes.
*Significantly different from control: $p<0.05$.

Table 4. Micronuclei induced by KA in the liver of young mice

| Chemical | Dose (mg/kg) | MNHPCs/1000HPCs (Mean±SEM) | | |
|---|---|---|---|---|
| | | 72 h | 96 h | 120 h |
| KA | 0 | 0.75±0.48 | 1.75±0.75 | 1.5±0.65 |
| | 500 | 0.75±0.25 | 2.00±0.82 | 1.75±0.75 |
| | 1000 | 2.00±0.41 | 2.00±0.41 | 2.75±0.75 |
| Cyclophosphamide | 20 | | 8.75±1.38* | |

MNHPCs, micronucleated hepatocytes; HPCs, hepatocytes.
*Significantly different from KA 0 control: $p<0.05$ by student $t$ test.

## Discussion

In the present study, KA samples used as a reagent, food additive and cosmetics ingredient showed mutagenicity in *S. typhimurium* TA100. The mutagenic activities of the samples were almost the same with or without S9 mix. The specific activity was weak, being around 100 revertants per mg (Table 2). The major, 270 nm absorbing component of each sample was isolated by preparative HPLC at a retention time of 8.5–10.5 min (Fig. 3b) and identified as KA by NMR analysis. Each KA sample supplied contained some UV absorbing materials which eluted later than KA (indicated by arrow in Fig. 3b). However, the impurity fraction, fraction 11, did not show any mutagenicity. When the mutagenic activities per unit weight of the three lots of KA supplied are compared with those of the major peak fractions 6–9 obtained by HPLC separation, there are no appreciable differences (Table 2). Although preparation methods of these three KA samples supplied are not available, features of these three samples are different and it is suspected that their preparation methods are different. Although a possibility of contamination of mutagenic non-UV absorbing material(s) which behave in the same way as KA on HPLC could not be completely negated, this possibility can be expected as negligible. Thus, it is indicated that all three lots did not contain mutagenic contaminants, and the mutagenic activity of the KA samples is solely derived from KA.

KA, at a dose of 1 g/kg, induced MN in the regenerating liver of mice. Thus, it seems that KA is genotoxic to liver. However this genotoxicity of KA was not detected in the liver of infant mice, on which partial hepatectomies were not performed. The reason for not detecting genotoxicity of KA in infant mice might have been because, while the mitotic index is expected to rise to a very high level after partial hepatectomy, it would be much lower in infant mice (3 weeks old). Further, differences in the metabolisms of infant mice and adult mice having received partial hepatectomies may play

some roles in this difference in genotoxicity (18). It is also noteworthy that KA did not induce MN in bone marrow of mice (12,13).

In contrast, KA was not genotoxic in regenerating rat's liver (Table 3). KA was also recently reported by Suzuki *et al.* (6). not to induce MN in young rats (4 weeks old). However, KA induced MN in peripheral blood of young rats (6). At present, neither the molecular mechanism of the genotoxicity of KA, nor the difference in metabolisms between rats and mice is known.

During preparation of this manuscript it has been reported in a two step carcinogenesis study, in which mice were fed a diet containing 3% KA at initiation step and phenobarbital was used as a tumor promoter, that there is no liver tumor initiating activity (19). At present, it is not clear whether the partial hepatectomy which was performed two weeks after the beginning of phenobarbital administration was at an appropriate time to detect initiating activity of KA or not. It is possible that the difference in the timing of the partial hepatectomy after cessation of KA administration might be one of the reasons for the discrepancy between KA being positive in MN induction and negative in initiating activity.

Although in the present study, genotoxicity of KA was detected in the mouse liver, it was not proved that this genotoxicity is involved in hepatic tumor development in mice.

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# Detection of 4-oxo-2-hexenal, a novel mutagenic product of lipid peroxidation, in human diet and cooking vapor

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**Abstract**

Since the diet plays an important role in the development of human cancer, it is important to identify mutagens in foods. We have detected a novel mutagenic product, 4-oxo-2-hexenal (4-OHE), in a model lipid peroxidation reaction mixture [H. Kasai, M. Maekawa, K. Kawai, K. Hachisuka, Y. Takahashi, H. Nakamura, R. Sawa, S. Matsui, T. Matsuda, 4-Oxo-2-hexenal, a mutgen formed by ω-3 fat peroxidation, causes DNA adduct formation in mouse organs, Ind. Health 43 (2005) 699–701]. In the present study, the contents of 4-OHE in various food samples were determined by a GC/MS method. Commercial perilla oil (derived from the seed of *Perilla frutescens* var. *frutescens*), which is rich in linolenic acid triglyceride (TG), the edible part of broiled fish, and various fried foods contained 4-OHE in the range of 1–70 μg/g. Furthermore, from the ethyl acetate trap (extracts) of the smoke released during the broiling of fish, 4-OHE was also detected by GC/MS. These results provide a warning to humans, who may be exposed to this mutagen. The 4-OHE may be produced from ω-3 polyunsaturated fats, such as α-linolenic acid-, docosahexaenoic acid (DHA)- and eicosapentaenoic acid (EPA)-TG, which are more easily oxidized than ω-6 fats, such as linoleic acid-TG.


*Keywords:* 4-oxo-2-hexenal; Lipid peroxidation; DNA adduct; Human diet; Cooking vapor

## 1. Introduction

It is well known that the human diet is an important factor in the causes of cancers. Especially, epidemiological studies have suggested that a high-fat diet and the consumption of red meat are risk factors of various cancers, such as breast, colon and prostate cancer. Lipid peroxyl radicals from oxidized oils and heme-iron were implicated as a mechanism of high-fat diet-induced colon carcinogenesis [1]. Lipid peroxidation products, such as 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal and 4-oxo-2-nonenal, reportedly modify DNA bases covalently [2–4]. However, there have been few studies on their detection in foods [5]. We have recently detected 4-oxo-2-hexenal (4-OHE)-deoxyguanosine (dG)-adduct, a novel nucleoside derivative, when dG was reacted with products in model lipid peroxidation reactions [6,7]. Although several studies have been carried out with lipid peroxidation-derived aldehydes, none of the reports have

*Abbreviations:* 4-OHE, 4-oxo-2-hexenal; 4-ONE, 4-oxo-2-nonenal; SIM, selected ion monitoring; SIM-TIC, SIM mode total ion chromatogram

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described the formation of 4-OHE. 4-OHE is probably generated by the oxidation of ω-3 fatty acids triglyceride (TG), which are commonly found in dietary fats, such as fish oil, perilla oil (derived from the seed of the plant *Perilla frutescens* var. *frutescens*, about 60% of perilla oil is α-linolenic acid TG), rapeseed oil, and soybean oil. Therefore, 4-OHE might be a major cytotoxic lipid peroxide in foods. We have also found that 4-OHE is mutagenic in the Ames test, and detected 4-OHE-DNA adduct formation in vivo [6,7]. Dietary exposure to 4-OHE may play an important role in human cancer development. Furthermore, epidemiological studies in China indicated that heated cooking oil vapors may be related to lung cancer risk [8,9]. Airborne 4-OHE produced by cooking may also be a risk factor for lung cancer in cooks [10,11]. In this study we determined the amounts of 4-OHE in food products and smoke condensates.

## 2. Materials and methods

### 2.1. Chemicals

4-Oxo-2-hexenal (4-OHE) diethylacetal was prepared by $MnO_2$ oxidation of 4-hydroxy-2-hexenal diethylacetal, which was synthesized according to the method of Esterbauer and Weger [12]. 4-OHE was obtained by acid treatment of 4-OHE diethylacetal. The structure of the synthetic 4-OHE thus obtained was confirmed by mass- and $^1H$ NMR-spectra. Detailed experimental conditions and spectral data will be published elsewhere [7]. Authentic 4-oxo-2-nonenal (4-ONE) was prepared by $MnO_2$ oxidation of 4-hydroxy-2-nonenal (a product of Calbiochem-Novabiochem Corp.; purchased from Wako Pure Chemical Ind. Ltd., Japan).

### 2.2. Ethyl acetate extracts of food

The food sample (1.0 g) was submerged in 2 ml of ethyl acetate in a screw-capped glass vial and extracted for 16 h at 4 °C. The resulting supernatant (40 μl) was diluted with 160 μl of ethyl acetate. The cooking oil was diluted 100-fold with ethyl acetate. These diluted solutions were used for the GC–MS analysis.

### 2.3. Smoke condensate

Fresh saury was obtained from a supermarket in Kitakyushu. One saury (about 180 g) was broiled until well-colored on a gas burner (about 8 min), using a grid. The smoke was collected by aspiration, using a funnel connected to a gas washing bottle filled with 100 ml of ethyl acetate. The smoke components were thus trapped by bubbling into ethyl acetate. The ethyl acetate extract was concentrated to a small volume under reduced pressure before the analysis by GC/MS.

### 2.4. Analytical methods

The standard 4-OHE solution in ethyl acetate (1 mg/ml) was diluted to various concentrations with ethyl acetate, and 2 μl of each was injected into the GC–MS apparatus. The calibration curve was obtained by the least square method. An authentic sample of 4-ONE was also analyzed by GC–MS, although the exact concentration was not determined due to the scarcity of the synthetic product. From each mass spectrum of 4-OHE and 4-ONE obtained by scanned GC–MS, the molecular ions ($M^+$, 112 and 154, respectively) and the major fragment ions ($M$–CHO, 83 and 139, respectively) were chosen as the identification ions for the GC–MS analysis in the selected ion monitoring (SIM) method. For SIM mode total ion chromatography (SIM-TIC), the identification ions at $m/z$ 112, 154, 83 and 139 were used. 4-OHE and 4-ONE were identified by their retention times and by detecting the identification ions ($M^+$, $M$–CHO). The concentrations of 4-OHE were determined by use of a calibration curve, which was produced on the basis of the ion counts of the molecular ions ($M^+$). The relative amount of 4-ONE was estimated from the ratio of the molecular ion count of 4-ONE to that of 4-OHE obtained by the SIM–GC–MS analysis, based on the assumption that homologous compounds have similar dose-ion count responses in the calibration curve [13]. Knowing the 4-OHE concentration, we thus estimated the 4-ONE concentration.

The diluted ethyl acetate extracts of food samples were transferred into a 200 μl glass vial and sealed with gas-tight caps. For the analysis of 4-OHE and 4-ONE, 2 μl aliquots were injected into GC–MS.

### 2.5. GC–MS conditions

The GC–MS analysis was carried out on a Hewlett-Packard gas chromatograph (HP 6890) connected to a mass spectrometer (JEOL JMS-BU 20, Tokyo). A 30 m × 0.25 mm i.d. CP-CIL5CB capillary column with a 250 μm film thickness (Chromopack) was used for the sample separation. The injection temperature was 280 °C. The column oven temperature was programmed at an initial temperature of 60 °C for 1 min, then to rise from 60 to 150 °C at a rate of 10 °C/min., and finally to increase from 150 to 270 °C at a rate of 40 °C/min. The flow rate of the carrier gas (He) was 1.5 ml/min (splitless). The MS analysis was carried out using electron impact ionization at 70 eV. The temperatures of the interface and ion chamber were 280 and 270 °C, respectively.

## 3. Results

The mass spectrum of 4-OHE, measured by our GC–MS, is shown in Fig. 1. The molecular ion ($M^+$) at $m/z$ 112 and the major fragment ion at $m/z$ 83 ($M$–CHO) are visible. Consequently, 4-OHE was determined by a SIM method at $m/z$ 112 (quantification ion) and confirmed by SIM at $m/z$ 83 (data not shown). The detection

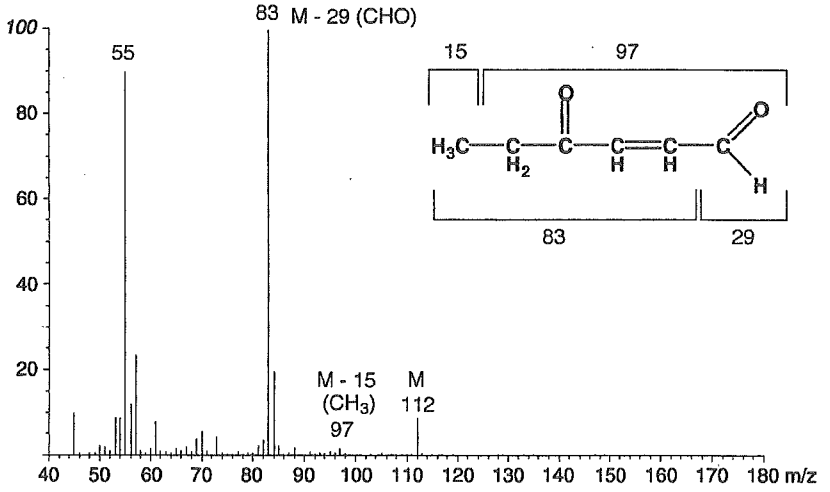


Fig. 1. Mass spectrum and specific mass fragments of 4-OHE.

limit was 0.01 μg/ml (S/N > 3), and the lower limit for the quantitative analysis was 0.05 μg/ml (S/N > 5).

The mass spectrum of 4-ONE (Fig. 2) shows the molecular ion at *m/z* 154 ($M^+$) and the major fragment ions at *m/z* 125 (*M*–CHO) and 139 (*M*–$CH_3$). For the semi-quantitative determination of 4-ONE, the SIM method at the molecular ion *m/z* 154 was used and confirmed by *m/z* 139, an associate ion for identification (data not shown).

Fig. 3A shows the results of the GC–MS analysis for the 4-OHE and 4-ONE present in broiled saury, as a typical example. It shows a SIM mode total ion chromatogram (SIM-TIC) and the mass chromatograph (SIM) at *m/z* values of 112 (the molecular ion) for 4-OHE and 154 (the molecular ion) for 4-ONE. Peaks 1 and 2 in Fig. 3 correspond to 4-OHE and 4-ONE, respectively. This was confirmed by the GC–MS chromatograms of authentic 4-OHE (Fig. 3B, B-1, B-2) and 4-ONE (Fig. 3B, B-3, B-4).

Table 1 summarizes the data for the 4-OHE contents in various foods. Higher levels of 4-OHE were found in broiled sardine and mackerel, than in the same fishes cooked by other methods. Furthermore, relatively high levels of 4-OHE were also observed in fried vegetables. On the other hand, most of the cooked meat samples did not contain detectable levels of 4-OHE, with the one exception of the beef fried in perilla oil. Concerning 4-ONE, since a highly pure synthetic standard is not available, we could not fully quantitate the amount of 4-ONE in food. However, by the ratio of the molecular ion counts of 4-OHE and 4-ONE, their approximate relative amounts were estimated. As a result, lower levels

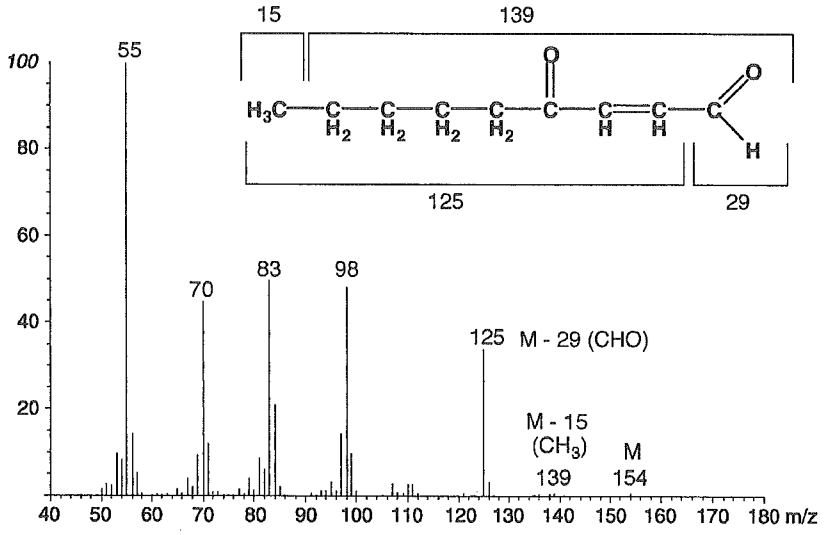


Fig. 2. Mass spectrum and specific mass fragments of 4-ONE.

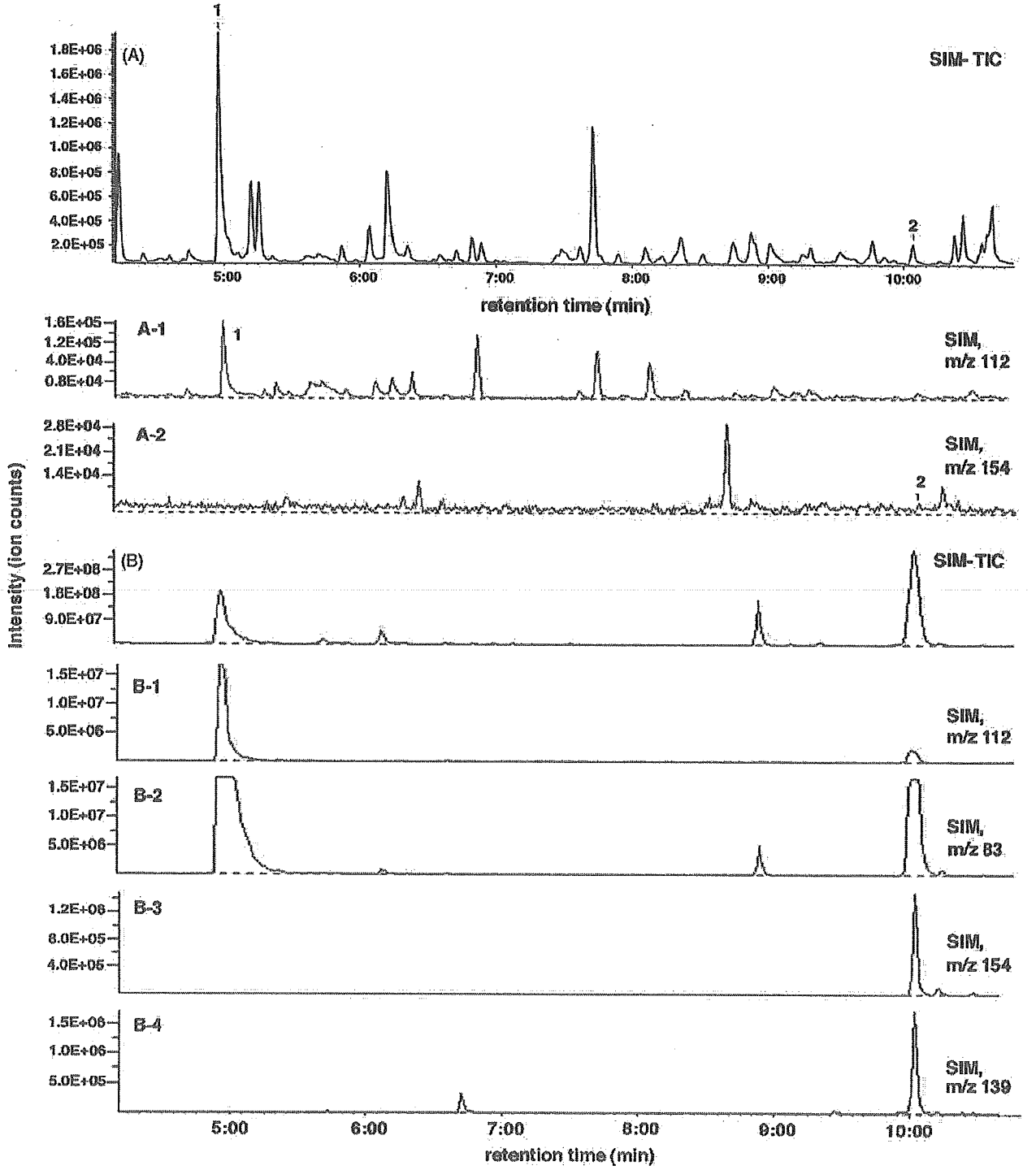


Fig. 3. (A) SIM-TIC and the mass chromatogram (SIM) of *m/z* 112 (A-1) and *m/z* 154 (A-2) of the ethyl acetate extract from broiled saury. Quantifications of peak 1 for 4-ONE ($t_R$ = 4:58, *m/z* 112), and peak 2 for 4-OHE ($t_R$ = 10:04, *m/z* 154). (B) SIM-TIC and mass chromatogram (SIM) of the mixture of authentic 4-OHE and 4-ONE. Identification of the peaks for 4-OHE ($t_R$ = 4:58, *m/z* 112: quantification ion and 83: identification ion) (B-1, B-2), and 4-ONE ($t_R$ = 10:04, *m/z* 154: quantification ion and 139: identification ion) (B-3, B-4).

of 4-ONE were detected, as compared to those of 4-OHE (Table 1).

The amount of 4-OHE in the smoke condensate obtained by broiling one saury was 31 μg. This value may be underestimated, because a portion of the 4-OHE is lost during the evaporation of ethyl acetate under reduced pressure. In contrast, 4-ONE was not detected in the smoke condensate.

Table 1
Amounts of 4-OHE and 4-ONE in cooked foods

| Food | Cooking method | 4-OHE (μg/g) | 4-ONE[a] |
|---|---|---|---|
| Cooking oil | | | |
| Perilla oil A | | 43.5 | N.D.[b] |
| Perilla oil B | | N.D. | N.D. |
| Fish | | | |
| Sardine (dried) | Broiled | 14.97 | N.D. |
| Sardine | Fritter (salad oil) | 7.09 | N.D. |
| | Boiled | 8.54 | N.D. |
| Salmon | Sauteed (salad oil, butter) | 3.67 | 1/5 |
| Saury | Broiled | 1.09–70.18 ($n$=8, mean 13.86) | N.D. |
| Mackerel | Broiled | 12.12 | 1/30 |
| | | 4.29 | N.D. |
| | Fried (salad oil) | 2.56 | N.D. |
| | Boiled | 5.49 | N.D. |
| Meat | | | |
| Chicken | Char-grilled | N.D. | N.D. |
| Pork | Fried (salad oil) | N.D. | N.D. |
| | Char-grilled | N.D. | N.D. |
| Beef | Fried (beef tallow oil) | N.D. | N.D. |
| | Fried (canola oil) | N.D. | N.D. |
| | Fried (salad oil) | N.D. | N.D. |
| | Fried (perilla oil A) | 45.88 | 1/60 |
| | Fried (perilla oil B) | N.D. | N.D. |
| | Fried (seasoned) | N.D. | N.D. |
| Vegetable | | | |
| Spinach | Fried (perilla oil A) | 5.28 | 1/5 |
| | Fried (perilla oil B) | 2.01 | 1/2 |
| | Fried (salad oil) | 4.40 | 1/2 |
| | Fried (canola oil) | N.D. | N.D. |
| Squash | Fried (salad oil) | 2.08 | N.D. |
| Other | | | |
| Snack (broad bean) | | N.D. | N.D. |
| Snack(corn) | | N.D. | N.D. |

[a] Approximate relative amount of 4-ONE to 4-OHE (molar ratio).
[b] N.D.: not detected.

## 4. Discussion

Our results represent the first description of the 4-OHE contents in food. Recently, 4-OHE was identified as a dG adduct in a dietary lipid peroxidation model (methyl linolenate + hemin) with dG [6,7]. We have also found that 4-OHE is mutagenic in the *Salmonella typhimurium* assay [6,7]. Furthermore, 4-OHE-DNA adducts were detected in the mouse gastrointestinal tract after oral administration of 4-OHE [6]. The 4-OHE may play an important role in human cancer development. From the aforementioned observations, it is important to determine the 4-OHE contents in food. In this study, we found that the amount of 4-OHE in most of the cooked fish was in the range of 2.6–70.2 μg/g food, though the amount was lower in cooked meat (barely detected). As an exception, we found an abundance of 4-OHE in fried beef, with frying performed in perilla oil (A). Actually, the perilla oil (A) itself contained a high amount of 4-OHE. The amounts of 4-OHE in food seem to correlate with the contents of ω-3 fats. Since 4-OHE is produced by the oxidation of α-linolenic acid TG, but not from linoleic acid TG, the 4-OHE may be formed by the oxidation of ω-3 fats. The presence of Fe or Cu in food ingredients may stimulate the formation of 4-OHE during cooking or preservation. It is well known that fish and perilla oil contain high levels of ω-3 fats. In addition, Sawa et al. [1] reported that lipid peroxyl radicals are formed from vegetable oils in the presence of heme-iron.

On the other hand, 4-hydroxy-2-nonenal (4-HNE) is the most widely studied aldehydic lipid peroxidation

product. 4-HNE is known to show cytotoxic effects. Recently, it was reported that 4-ONE is also a major product of lipid peroxidation [14]. In addition, 4-ONE is more reactive than 4-HNE toward DNA. Therefore, we also measured the amounts of 4-ONE in foods, and found that the levels of 4-ONE were lower than those of 4-OHE. This result is consistent with the fact that we did not detect the 4-ONE-dG adduct in a model reaction consisting of a mixture of methyl linoleate, hemin and dG (data not shown). As mentioned previously, 4-OHE is a lipid peroxidation product derived from ω-3 fats. In contrast, 4-ONE is produced from the ω-6 series of fats. The detection of a higher amount of 4-OHE than 4-ONE is also consistent with the fact that there are more ω-3 fats, such as α-linolenic acid-, docosahexaenoic acid (DHA)- and eicosapentaenoic acid (EPA)-TG, than ω-6 fats, such as linoleic acid TG, in most of the food samples we measured. Another explanation for the higher level of 4-OHE than 4-ONE is that the major ω-3 fats, α-linolenic acid-, EPA- and DHA-TG, in food are more rapidly oxidized than the major ω-6 fat, linoleic acid TG.

In the case of heterocyclic amines, which are well known carcinogens in the human diet, the most abundant, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), has been detected in cooked food in the range of 0.3–180 ng/g [15]. In comparison, the amounts of 4-OHE in the human diet are two to three orders of magnitude higher than those of heterocyclic amines.

Epidemiological [8,9] and experimental [10,11,16] studies have demonstrated that cooking oil vapors may be related to cancer risk. Some airborne samples taken in the cooking areas were mutagenic to the TA 98 and TA 100 Salmonella strains without metabolic activation [17]. Since 4-OHE was detected in the smoke condensate from saury and it is mutagenic in the Ames test without metabolic activation [6], it may play a role as a volatile mutagen produced during cooking, as well as a mutagen in food.

Recently, it was suggested that the ingestion of ω-3 fats has desirable effects on human health [18], by preventing cardiovascular disease and cancer. In contrast, some studies [19] suggested that α-linolenic acid TG intake might be a risk factor. These discrepancies may be attributable to the 4-OHE content, depending upon the extent (degree) of lipid peroxidation.

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# Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells

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**Abstract**

The recent finding that acrylamide (AA), a potent carcinogen, is formed in foods during cooking raises human health concerns. In the present study, we investigated the genotoxicity of AA and its metabolite glycidamide (GA) in human lymphoblastoid TK6 cells examining three endpoints: DNA damage (comet assay), clastogenesis (micronucleus test) and gene mutation (thymidine kinase (*TK*) assay). In a 4 h treatment without metabolic activation, AA was mildly genotoxic in the micronucleus and *TK* assays at high concentrations (>10 mM), whereas GA was significantly and concentration-dependently genotoxic at all endpoints at ≥0.5 mM. Molecular analysis of the *TK* mutants revealed that AA predominantly induced loss of heterozygosity (LOH) mutation like spontaneous one while GA-induced primarily point mutations. These results indicate that the genotoxic characteristics of AA and GA were distinctly different: AA was clastogenic and GA was mutagenic. The cytotoxicity and genotoxicity of AA were not enhanced by metabolic activation (rat liver S9), implying that the rat liver S9 did not activate AA. We discuss the in vitro and in vivo genotoxicity of AA and GA.


*Keywords:* Acrylamide; Gycidamide; Genotoxicity; *TK* mutation; Metabolic activation

## 1. Introduction

Acrylamide (AA) is a synthetic chemical that has been produced since the early 1950s. Because AA polymerizes easily to an adhesive gel, it has been widely used in industry for water flocculation, soil coagulation and grouts. Because it had been believed that humans are rarely exposed to AA under ordinary circumstances, concern was centered only on occupational exposure [1]. In 2000, however, Tareke et al. [2] reported that AA was unexpectedly discovered in cooking foods. It forms during frying and baking principally by a Maillard reaction between asparagine residues and glucose [3,4]. This finding raises concerns about the health risks of AA for the general population [5].

According to toxicological studies, AA is neurotoxic for animals and human [6,7], and the International

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Agency for Research on Cancer classifies it as 2A, a probable human carcinogen [1]. AA is also genotoxic in somatic and germinal cells in in vitro and in vivo [8]. In vivo examination [8] AA is metabolized to the epoxide derivative glycidamide (GA), presumably by cytochrome P4502E1 (CYP2E1) [9]. GA may be more toxic than AA because it reacts quickly with DNA and other biological macromolecules, and it is positive in most genotoxicity tests [8]. AA, on the other hand, is inactive in bacterial and some in vitro mammalian gene mutation assays, but it induces sister chromatid exchanges and chromosome aberrations in vitro and in vivo [8]. AA may have indirect genotoxic mechanisms, such as protein binding, spindle disturbance or hormonal imbalance, which could lead to tumors [10,11]. Thus, the genotoxic mechanism of AA is unclear.

In the present study, we used human lymphoblatoid TK6 cells to investigate the genotoxicity of AA and GA and its mechanisms. TK6 cells are widely used for the thymidine kinase (*TK*) gene mutation assay and can also be used in the in vitro micronucleus (MN) and comet (COM) assays. The *TK* gene mutation assay detects a wide range of genetic damage, including gene mutations, large-scale chromosomal deletions, recombination and aneuploidy [12], while other mammalian gene mutation assays, such as the *HPRT* and transgenic *LacZ* and *LacI* gene assays, detect only point mutations and small deletions [13]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumably relevant to carcinogenesis. Molecular analysis of the TK mutants induced by AA or GA can help elucidate their genotoxic mechanisms. In addition, because it uses a human cell line, the *TK* assay is appropriate for human hazard evaluation.

## 2. Materials and methods

### 2.1. Cell culture, chemicals and treatment

The TK6 human lymphoblastoid cell line has been described previously [14]. The cells were grown in RPMI1640 medium (Gibco-BRL, Life technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at $10^5$ to $10^6$ cells/ml at 37 °C in a 5% $CO_2$ atmosphere with 100% humidity.

AA (CAS # 79-06-1) and GA (CAS # 5694-00-8) were purchased from Wako Pure Chemical Co. (Tokyo). We dissolved them in phosphate-buffered saline just before use. *N*-di-*N*-butylnitrosamine (DBN) (CAS # 924-16-3) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo) and dissolved in DMSO for use. Post-mitochondrial supernatant fractions of liver homogenate (S9) were purchased from Kikkoman Co. Ltd. (Noda, Chiba, Japan), which were prepared from the liver of phenobarbital- and 5,6-benzoflavone-treated SD rats. We prepared a 10 ml S9 mix with 4 ml S9 fraction and 2 ml each of 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP and 150 mM KCl.

We treated 20 ml aliquots of cell suspensions ($5.0 \times 10^5$ cells/ml) at 37 °C for 4 h with serially diluted AA or GA, washed them once, re-suspended them in fresh medium, and cultured them in new flasks for the MN and *TK* assays or diluted and plated them for survival measurement (PE0). We treated the cultures with AA both in the absence and presence of 5% S9 mix.

### 2.2. Comet assay

After treating the cells for 4 h with AA or GA, we prepared slides for alkaline COM assay as previously reported [15]. Briefly, the cells were suspended in 0.5% agarose-LGT (Nakalai Tesque Inc., Kyoto, Japan), quickly layered on a slide (Matsunami Glass Ind. Ltd., Osaka, Japan) coated with 1% agarose GP-42 (Nakalai Tesque Inc.), and covered with 0.5% agarose-LGT. We immersed the slide in alkaline lysing solution (pH 13) for 1 h, electrophoresed it for 15 min after the unwinding treatment, fixed the cells with 70% ethanol, and stained them with SYBER green (Molecular Probes, Eugene, OR) according to the manufacturer's recommendation. We observed the cells by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and the tail length of the comet image was measured. We statistically analyzed the difference between the non-treated and treated plates with the Dunnett's test after one-way ANOVA [16].

### 2.3. Micronuclei test

Forty-eight hours after treatment, we prepared the MN test samples as previously reported [17]. Briefly, approximately $10^6$ cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold glacial acetic acid in methanol (1:3), and resuspended in methanol containing 1% acetic acid. We placed a drop of the suspension on a clean glass slide and allowed it to air-dry. We stained the cells with 40 μg/ml acridine orange solution and immediately observed them by Olympus model BX50 fluorescence microscope. At least, 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test. The concentration–response relationship was evaluated by the Cochran–Armitage trend test [18].

### 2.4. TK gene mutation assay

The TK6 cell cultures were maintained for 3 days after treatment to permit expression of the *TK* deficient phenotype. To isolate the *TK* deficient mutants, we seeded cells from each

culture into 96-microwell plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluorothymidine (TFT). We also plated them at 1.6 cells/well in the absence of TFT for the determination of plating efficiency (PE3). All plates were incubated at 37 °C in 5% $CO_2$ in a humidified incubator. The TK assay produces two distinct phenotypic classes of *TK* mutants: normally growing (NG) mutants had the same doubling time (13–17 h) as the wild type cells, and slowly growing (SG) mutants had a doubling time of >21 h. The difference is thought to be due to a putative gene near the *TK* gene. NG mutants result mainly from intragenic mutations, such as point mutations and small deletions, while SG mutants result from gross genetic changes extending beyond the *TK* gene [19]. We scored for the colonies in the PE plates and for the colonies for normal-growing *TK* mutants in the TFT plates at 14th day after plating. We then refed the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing *TK* mutants. Mutation frequencies were calculated according to the Poisson distribution [20]. The data were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson–Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [21].

### 2.5. Molecular analysis of TK mutants

Genomic DNA was extracted from *TK* mutant cells and used as a template for the polymerase chain reaction (PCR). We analyzed for loss of heterozygosity (LOH) at the human *TK* gene by PCR products as described previously [22]. A set of primers was used to each amplify the parts of exons 4 and 7 of the *TK* gene that contains frameshift mutations. Another primer set for amplifying parts of the β-globin were also prepared. We used quantitative-multiple PCR to co-amplify the three regions and to identify and quantify the PCR products. We analyzed them with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and classified the mutants into "none LOH", "hemizygous LOH" or "homozygous LOH". To determine the extent of LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis described previously [22]. The results were processed by GenoTyper™ software (PE Biosystems) according to the manufacturer's guidelines.

## 3. Results

### 3.1. Cytotoxic and genotoxic responses to AA and GA

Fig. 1a shows the effect of AA on relative survival (RS), mutation frequency (*TK* assay) and number of micronucleated cells per 1000 cells examined. AA was concentration-dependently cytotoxic, permitting about 20% RS at the maximum concentration (14 mM), while its genotoxicity and clastogenicity were weak. We repeated the experiment because of the weak genotoxicity. AA showed negative in the first *TK* assay, but positive in the second statistically. In MN test, both experiments showed statistically positive. GA, in contrast, was significantly genotoxic even at concentrations that were not severely cytotoxic (Fig. 1b). At the maximum concentration (2.4 mM), GA induced *TK* mutation frequencies that were about 20 times and MN fre-

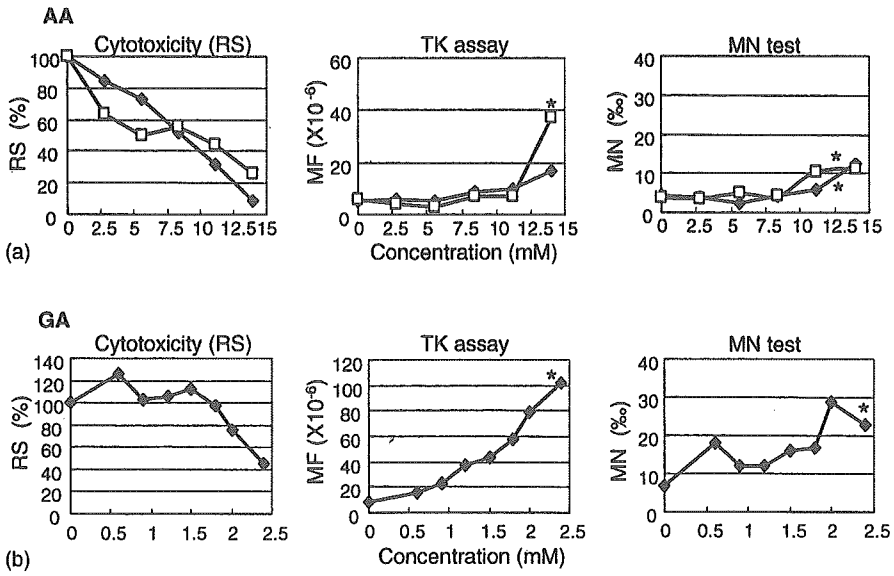


Fig. 1. Cytotoxic (relative survival, RS), genotoxic responses (TK assay and MN test) of TK6 cells treated with AA (a) or GA (b) for 4 h without metabolic activation. The AA experiment was repeated to confirm the result because of the weak genotoxicity. Closed and open symbols are first and second experiment, respectively. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

Table 1
Cytotoxic and mutational responses to AA and GA, and the results of LOH analysis of normally growing (NG) and slowly growing (SG) TK-mutants

| Treatment | Cytotoxic and mutational response | | | LOH analysis at TK gene | | | |
|---|---|---|---|---|---|---|---|
| | RS (%) | MF ($\times 10^{-6}$) | % SG | No. | None LOH | Hemi-LOH | Homo-LOH |
| Vehicle [16] | 100 | 2.19 | 56 | 56 | | | |
| NG mutants | | | | 19 | 14(74) | 3(16) | 2(11) |
| SG mutants | | | | 37 | 0(0) | 9(24) | 28(76) |
| AA (14 mM, 4 h) | 40 | 18.9 | 54 | 48 | | | |
| NG mutants | | | | 22 | 11(50) | 11(50) | 0(0) |
| SG mutants | | | | 26 | 0(0) | 13(50) | 13(50) |
| GA (2.2 mM, 4 h) | 12 | 55.5 | 36 | 44 | | | |
| NG mutants | | | | 28 | 26(93) | 2(7) | 0(0) |
| SG mutants | | | | 16 | 0(0) | 6(38) | 10(62) |

quencies at about four times the spontaneous level. We detected two distinct phenotypic classes of *TK* mutants in *TK* assay: NG and SG mutants. AA did not affect the proportion of SG mutants, while GA treatment lowered it (Table 1). This implies that GA induced primarily point mutations. In the COM assay, even at the highest concentration, AA did not induce DNA damage, while GA did so strongly starting at 0.6 mM (Fig. 2).

### 3.2. Molecular analysis of TK mutants

The *TK* mutants were independently isolated from the cells treated with 14 mM AA or 2.2 mM GA for 4 h. Table 1 shows the cytotoxicity (RS) and *TK* mutation frequency (MF) and proportion of SG mutants (% SG) by the treatment. Genomic DNA extracted from the mutants was subjected by the PCR-based LOH analysis to classify the mutants into three types: non-LOH, hemizygous LOH (hemi-LOH) and homozygous LOH (homo-LOH). In general, hemi-LOH is resulted by deletion and homo-LOH is by inter-allelic homologous recombination [13]. We analyzed 48 AA-induced and 44 GA-induced *TK* mutants and compared them to those of spontaneously occurring *TK* mutants described previously [16]. The fraction of hemi-LOH in AA-induced mutants, in which 50% each of NG and SG mutants exhibited hemi-LOH, was higher than in spontaneous mutants, indicating that AA-induced primarily deletions. GA, on the other hand, induced primarily NG mutants, and most (93%) of them were the non-LOH type, which is presumably generated by point and other small intragenic mutations. Among 16 GA-induced SG mutants, the percentages that were hemi-LOH (38%) and homo-LOH (62%) were similar to those observed in spontaneous SG mutants. Fig. 3 shows the mutation spectra of *TK* mutants found among treated and untreated TK6 cells. GA and ethyl methane sulfonate, an alkylating agent, produce similar spectra, as do AA and X-radiation.

Fig. 4 shows the distribution of LOH in AA-induced ($n = 37$), GA-induced ($n = 17$) and spontaneous ($n = 29$) LOH mutants. Because the majority of GA-induced mutants were the non-LOH type, we were able to map only 17 GA-induced LOH mutants. As a particular characteristic of AA-induced LOH mutants, we frequently observed small deletions limited to the *TK* locus. The

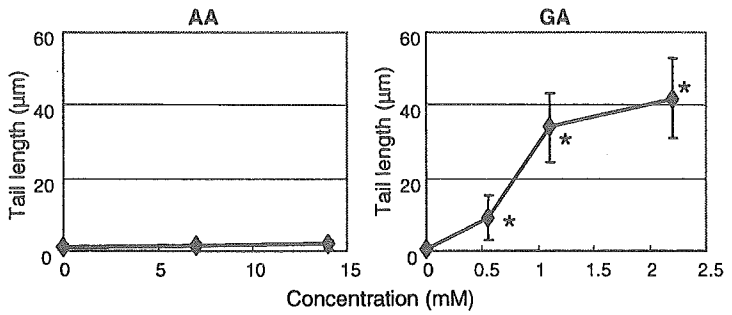


Fig. 2. COM assay results in TK6 cells treated with AA or GA for 4 h without metabolic activation. Asterisk (*) statistically significant in the Dunnett's tests ($P < 0.05$).

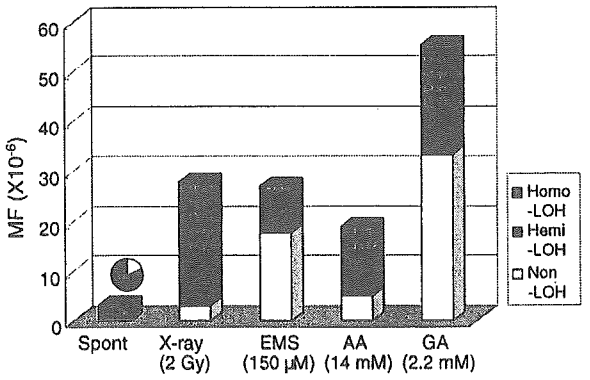


Fig. 3. Frequency and spectra of TK mutations in spontaneous and X-ray-induced (2 Gy), EMS-induced (150 μM, 4 h), AA-induced (14 mM, 4 h) and GA-induced (2.2 mM, 4 h) TK mutants in TK6 cells. The fraction of each mutational event was calculated by considering the ratio of normally growing (NG) and slowly growing (SG) mutants and the results of molecular analysis (Table 1). The data of spontaneous, X-ray-induced and EMS-induced mutation spectra were taken from our previous paper [13].

distribution of LOH in GA-induced and spontaneous LOH mutants was similar.

### 3.3. Cytotoxicity and genotoxicity of AA under metabolic activation

Rat liver S9 mix did not influence the cytotoxicity or genotoxicity of AA but it did enhance the activity of DBN, the positive control chemical (Fig. 5).

## 4. Discussion

A large number of studies about the in vitro genotoxicity of AA have been reported [8]. AA has consistently been negative in bacterial gene mutation assay in both the presence and absence of metabolic activation [23–25] but positive in chromosome aberration and sister chromatid exchange tests in Chinese hamster cell lines [24–26]. In mammalian cell assays, AA induces *Tk* but not *Hprt* gene mutations [24,25,27,28], and is negative in the COM assay even at high concentrations [27]. These results suggest that AA is clastogenic without directly damaging DNA. GA, on the other hand, is positive in most in vitro genotoxicity tests and is recognized as a mutagen [8,27,29]. In the present study, the higher concentrations of AA were positive in the MN and TK assay but negative in the comet assay. According to the in vitro genotoxicity test guideline, however, AA may be negative [30], because the guideline suggests that the maximum concentration should be 10 mM. Because the genotoxic responses at higher concentrations were reproducible, AA may be genotoxic, but its effect is very weak. GA, in contrast, was positive in all the assays, even under conditions of low cytotoxicity. These results are consistent with the reports described above.

The mammalian *TK* gene mutation assay can detect a wide range of genetic changes, including point mutations, small deletions, large-scale chromosomal deletions, inter-allelic recombination and aneuploidy, while

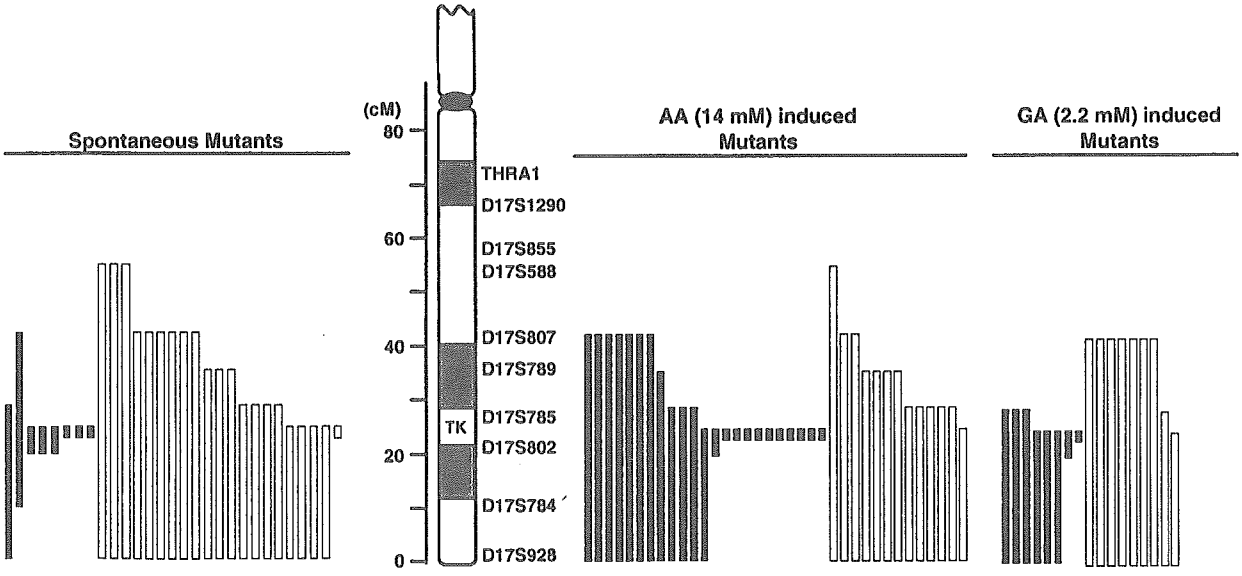


Fig. 4. The extent of LOH in spontaneous, AA-induced and GA-induced LOH mutants from TK6 cells. We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human TK locus maps to 17q23.2. Open and closed bars represent homo-LOH and hemi-LOH, respectively. The length of the bar indicates the extent of the LOH. We analyzed 29 spontaneous mutants (10 NG and 19 SG mutants), 37 AA-induced mutants (11 NG and 26 SG) and 17 GA-induced mutants (2 NG and 15 SG). The data on spontaneous mutants were taken from our previous paper [13].

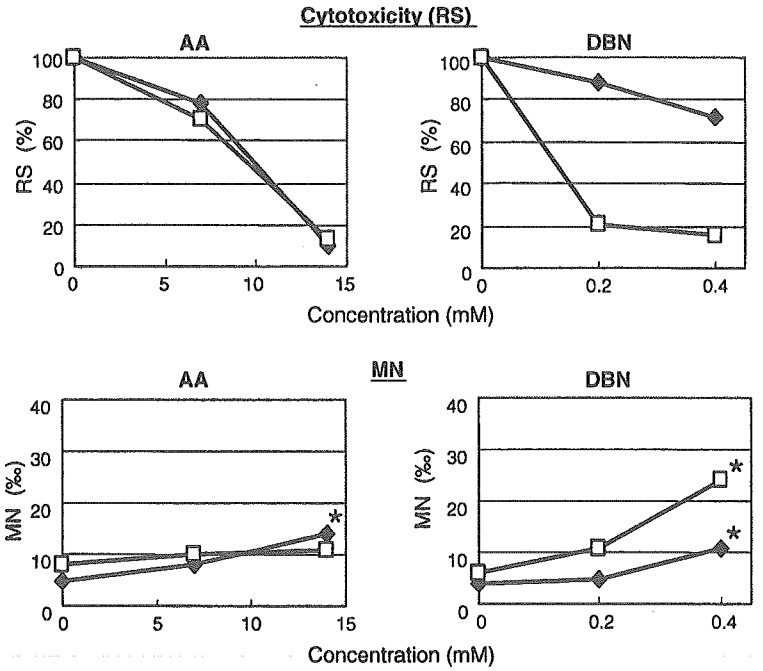


Fig. 5. Cytotoxicity (RS) and MN induction in TK6 cells treated with AA or DBN for 4 h in the presence (open symbol) or absence (closed symbol) of rat liver S9. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

the bacterial and mammalian *HPRT* gene mutation assays detect only point mutations and small intragenic deletions [13]. AA was positive only in the *TK* mutation assay, suggesting that AA causes predominantly large-scale chromosomal changes. Our molecular analysis of the *TK* mutants supported this hypothesis. The majority of the AA-induced *TK* mutants showed hemi-LOH, which is the result of a deletion, although the other types were also induced (Fig. 3). Deletions are thought to result from the repair of double strand breaks by non-homlogous end-joining [13]. Radiation-induced double strand breaks are repaired by non-homlogous end-joining, which leads to hemi-LOH. LOH-mapping analysis, however, revealed that AA frequently induces intermediate-sized deletions (100–3000 kb); the deletions encompass exons 4 and 7 of the *TK* locus but do not extend to the microsatellites loci of the vicinity. This type of deletion is rarely observed in radiation-inducing *TK* mutants [13]. Because the COM assay indicated that AA did not induce DNA damage, the deletion may not be caused by DNA damage directly. Mechanisms associated with global genomic instability should also be considered [10] because the LOH patterns, except for the intermediate-sized deletions, are generally similar to those observed in spontaneous mutants. Most GA-induced *TK* mutants, on the other hand, were the non-LOH type, as were most spontaneous ones, strongly supporting the positive results in bacterial gene mutation assay [29]. In contrast to AA, GA is a mutagen, inducing primarily point mutations.

AA is known to be metabolized to GA by CYP2E1 [9]. GA, an epoxide, forms adducts directly with DNA and protein, causing cytotoxicity and genotoxicity. GA forms mainly *N*7-(2-carbamoyl-2-hydroxyethyl) guanine and *N*3-(2-carbamoyl-2-hydroxyethyl) adenine and reacts with hemoglobin and cytoskeletal proteins [31–33]. Rat S9, however, did not affect AA cytotoxicity or genotoxicity, although it did enhance the cytotoxicity and genotoxicity of DBN, which is also metabolized by CYP2E1. This suggests that rat S9 does not work for activating AA. AA and GA are detoxified through glutathione conjugation, and GA is also detoxified by epoxy hydrolase (EH), which catalyzes the hydrolysis of GA to dihidroxy propionamide [34,35]. Other in vitro studies also failed to demonstrate the enhancement of AA genotoxicity by rat S9 [36,37]. Our results do not mean that AA is always detoxified rather than activated because DNA adducts are found in mice and rats given oral AA, and the genotoxicity of AA is consistently observed in in vivo studies [8,31,36,37]. Recently, Manjanatha et al. demonstrated in transgenic Big Blue$^{TM}$ mice that AA as well as GA induces endogenous *Hprt* and transgenic *cII* mutation at same level, and both chemicals cause predominantly base substitutions and frameshift mutations.