Inhibitory Effects of Beer and Other Alcoholic Beverages on Mutagenesis and DNA Adduct Formation Induced by Several Carcinogens

Sakae Arimoto-Kobayashi,*,† Chitose Sugiyama,‡ Nanaho Harada,† Miyuki Takeuchi,† Miyuki Takemura,† and Hikoya Hayatsu†

Faculty of Pharmaceutical Sciences and Graduate School of Natural Science and Technology, Okayama University, Tsushima, Okayama 700-0082, Japan

The possibility that beer and other alcoholic beverages could be antimutagenic against the heterocyclic amines (HAs), a group of carcinogens produced on cooking proteinaceous foods, has been explored. In the *Salmonella* mutation assays, beer showed inhibitory effects against several HAs [preactivated Trp-P-1, Trp-P-2(NHOH), and Glu-P-1(NHOH)] that are directly mutagenic in bacteria. Japanese sake, red and white wines, and brandy were also effective. However, ethyl alcohol alone did not show these effects. The formation of O^6-methylguanine by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine in the DNA of *Salmonella* YG7108 was also inhibited by beer. Nonvolatile beer components were administered orally to CDF_1 mice together with Trp-P-2. Adducts in the liver DNA were significantly decreased by the beer, as compared to those in controls fed Trp-P-2 only. Although several phenolic compounds known to be present in beer were antimutagenic toward these mutagens, their effects were very small. It was concluded that some yet to be identified component(s) of beer is (are) responsible for this antimutagenicity.

Keywords: *Antimutagenicity; beer; alcoholic beverages; plant phenols; food pyrolysate mutagens; DNA adducts*

INTRODUCTION

Antimutagenicity and anticarcinogenicity of dietary components are being studied extensively (World Cancer Research Fund, 1997; Ohigashi et al., 1997). Epidemiological studies have demonstrated reduced mortality from coronary heart diseases among moderate alcohol consumers by comparison with abstainers (Rimm et al., 1991). On the other hand, the evidence that alcohol itself increases the risk of mouth, esophageal, and primary liver cancers appears convincing (World Cancer Research Fund, 1997). There are conflicting reports concerning the relationship between beer consumption and cancer risk. For example, Riboli et al. (1991) have reported that beer consumption is not associated with colon cancer, but Kato et al. (1990) have shown that beer drinkers have an increased risk to colorectal cancer. An inverse association between moderate beer consumption and endometrial cancer was suggested by Swanson et al. (1993). However, a relationship between beer consumption and lung cancer was suggested by Potter et al. (1992).

Nonvolatile dissolved materials in beer amount to as much as 5% (w/w), as observed in our laboratory on freeze-drying of beer samples. Therefore, for any biological effects of beer, these dissolved components should be taken into account. In this work, we explored the possibility that the dissolved components of beer and other alcoholic beverages might show antimutagenic and antigenotoxic properties. Several individual phenolic compounds in beer were also examined. In addition, we investigated whether beer can inhibit carcinogen-induced DNA adduct formation in vivo.

MATERIALS AND METHODS

Alcoholic Beverages. Various types of beer, for example, lager, stout, nonalcoholic, and ale, were used. In total, 24 different samples of lager beer were examined: 9 (lagers A−I) produced in Japan, 1 from South Africa (J), 1 from Italy (K), 1 from the United States (L), 1 from The Netherlands (M), 1 from Scotland (N), 1 from Germany (O), 1 from Russia (P), and 1 from France (Q); among them, samples F−I were "black beer". There were 4 samples of stout beer: 1 from Japan (stout A), one from the United States (B), 1 from England (C), and 1 from Ireland (D). One nonalcoholic beer (produced in the United States) and two ales (ale A from Scotland and ale B from England) were also investigated. Lagers E−G, K, and Q, nonalcoholic, ale A, and stouts A−C were bottled, and the rest were canned products. The purchase places were Cape Town (lager J), Essen (lager O), Moscow (lager P), Paris (lager Q), Boston (nonalcoholic and stout B), London (stout D), and Okayama (all the rest). We also purchased in Okayama 2 samples of red wine [1 from France (red wine A) and 1 from Chile (red wine B)], 3 samples of white wine [1 from Japan (white wine A), 1 from Germany (white wine B), and 1 from France (white wine C)], 2 samples of sake (A and B) made in Japan, 2 samples of brandy (A from France and B from Japan), 2 samples of whiskey (A from the United States and B from Scotland), and 2 samples of shochu (a product from barley) (A and B) from Japan. One sample of white wine made in Austria (white wine D) was bought in Vienna. Freeze-dried beer samples were used in some experiments.

Other Materials. The phenolic compounds, vanillic acid, syringic acid, protocatechuic acid, gentisic acid, gallic acid,

* Author to whom correspondence should be addressed (telephone 81-86-251-7947; fax 81-86-251-7927; e-mail arimoto@cc.okayama-u.ac.jp).

† Faculty of Pharmaceutical Sciences.

‡ Graduate School of Natural Science and Technology.

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4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid), caffeic acid, *p*-coumaric acid, phloroglucinol, and chlorogenic acid, were purchased from Nacalai Tesque (Kyoto, Japan). Hop was obtained from Kirin Brewery Co. (Tokyo, Japan). Ellagic acid, *p*-hydroxyphenethyl alcohol, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) [CAS Registry No. 12994-1 (all CAS Registry No. were supplied by the authors)] were purchased from Wako Chemicals (Osaka, Japan), and methylmethane sulfonate and 2-aminofluorene were from Aldrich Japan (Tokyo, Japan). Benzo[*a*]pyrene-7,8-diol 9,10-epoxide (BPDE) was obtained from the NCI Chemical Reference Standard Repositories (USA). 3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido-[4,3-*b*]indole [Trp-P-2 (CAS Registry No. 72254-58-1)], 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethyl-imidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP), and 2-aminofluorene were obtained from Wako Chemicals. O^6-Methylguanine was purchased from Sigma (St. Louis, MO). 3-Hydroxyamino-1-methyl-5*H*-pyrido-[4,3-*b*]indole [Trp-P-2(NHOH)] was synthesized from Trp-P-2 as described by Saito et al. (1983), and 2-(hydroxyamino)-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole [Glu-P-1(NHOH)] was synthesized according to the method of Hashimoto et al. (1982). These hydroxyamino derivatives were >99.5% pure as checked by high-performance liquid chromatography (HPLC). 2-(Hydroxyamino)-3-methylimidazo[4,5-*f*]quinoline [IQ(NHOH)] was prepared from IQ as described by Saito et al. (1983) and Snyderwine et al. (1987). The material obtained and used in the present experiments was a mixture of IQ(NHOH) (49.5%) and IQ (50.5%) as checked by HPLC, and the direct-acting mutagenicity found for this material was regarded as represented by the IQ(NHOH) in it. Direct-acting mutagens from Trp-P-1, IQ, MeIQ, MeIQx, PhIP, and 2-aminofluorene (hereafter referred to as activated Trp-P-1, etc.) were prepared according to the method described earlier (Arimoto et al., 1980): briefly, a mutagen in 10 mM sodium phosphate buffer at pH 7.4 was treated with S9 (see below) for 20 min at 37 °C, an equal volume of cold acetone was added to the solution, the soluble fraction of the mixture was separated and evaporated under reduced pressure, and the residue obtained was dissolved in cold water. These activated mutagen samples were stored at −80 °C until use. *Salmonella typhimurium* TA98 and TA100 were gifts from Dr. B. N. Ames of the University of California, Berkeley, and the strain YG7108 was a gift from Dr. M. Yamada of the National Institute of Hygienic Sciences, Tokyo (Yamada et al., 1995). For enzymatic activation, polychlorinated biphenyl (PCB-54, Cl content ∼54%, from Tokyo Kasei Chemical Co.)-induced rat S9 was used (Ames et al., 1975). CDF_1 mice (6 weeks old) were obtained from Charles River Japan (Atsugi, Japan).

Mutagenicity Assays and Inhibition Experiments. The preincubation method (Yahagi et al., 1977) was employed in the *Salmonella* mutagenicity assays (Ames et al., 1975). Modifying effects of compounds on the mutagenicity were examined by using the procedure described earlier for chlorophyllin (Arimoto et al., 1982). Briefly, the preincubation mixture was prepared by mixing the components in the following order: 100 μL of a solution of beer [or its component(s)] or an alcoholic beverage, 450 μL of 0.1 M sodium phosphate buffer at pH 7.4, 100 μL of an overnight culture of bacteria, and finally 50 μL of a mutagen solution. In all of these assays, ethanol concentrations in a series of experiments were fixed at desired values by supplementing appropriate amounts of ethyl alcohol into the reaction mixtures. Thus, the concentration of alcohol in the preincubation mixture was adjusted to 0.4−0.6% for the experiments of beer, 1% for wine and sake, 2.6% for brandy, 3% for whisey, and 1.8% for shochu. After incubation for 20 min at 37 °C, molten agar was added and the mixture was poured onto a minimal agar plate. The plates were incubated for 2 nights, and the revertant colonies that resulted were counted manually. The doses of individual mutagens used were those with which ≥600 revertant colonies per plate were formed. When the number per plate exceeded 3000, colonies in a certain square area were counted, and from the average counts in five such areas the total number on the plate was estimated. Mutagenic activity (percent) given in the figures is a value obtained as follows:

$$100 \times [(\text{His}^+ \text{ revertants in the presence of beer}) - (\text{spontaneous revertants})]/[(\text{His}^+ \text{ revertants in the absence of beer}) - (\text{spontaneous revertants})] \quad (1)$$

The numbers of His^+ revertants from *S. typhimurium* TA98 (−S9) per plate found in the absence of the inhibitor were 2575−3050 for 20 pmol of Trp-P-2(NHOH), 1050−1290 for 10 pmol of IQ(NHOH), 1390−1970 for 0.2 nmol equiv of activated Trp-P-1, 2550−2740 for 1 nmol of Glu-P-1(NHOH), 607−1330 for 20 nmol equiv of activated 2-aminofluorene, 2150−2060 for 2.5 nmol equiv of activated PhIP, 1010−1070 for 40 pmol equiv of activated IQ, 3180−3210 for 40 nmol equiv of activated MeIQ, and 1750−2560 for 0.1 nmol equiv of activated MeIQx; and those for *S. typhimurium* TA100 (−S9) were 1610−1350 for 4 nmol of MNNG, 1520−1590 for 0.2 μmol of BPDE, and 1020−1250 for 5 nmol of methylmethanesulfonate. The numbers of the spontaneously formed revertants were 20−43 with TA98 and 133−155 with TA100.

Sequential Treatment of *S. typhimurium* TA98 with an Inhibitor and Trp-P-2(NHOH). A 10-mL sample of an overnight culture of *S. typhimurium* TA98 was centrifuged at 15 000 rpm at 4 °C. The pellet obtained was suspended in 2 mL of phosphate-buffered saline. For pretreatment with an inhibitor, a mixture of 0.1 mL of the bacterial suspension, 0.5 mL of 0.1 M sodium phosphate (pH 7.4), and 0.1 mL of an inhibitor solution was incubated for 20 min at 37 °C. After the incubation, the mixture was centrifuged at 15 000 rpm at 4 °C. The separated pellet was resuspended in 0.6 mL of phosphate-buffered saline and mixed with 0.1 mL of 200 nM Trp-P-2(NHOH), and the mixture was incubated for 10 min at 37 °C before soft agar (2 mL) was added for plating.

For a post-treatment with an inhibitor, 0.1 mL of the bacterial suspension, 0.5 mL of 0.1 M sodium phosphate (pH 7.4), and 0.1 mL of 200 nM Trp-P-2(NHOH) were mixed, and the mixture was incubated for 20 min at 37 °C. After the incubation, the mixture was centrifuged at 15 000 rpm at 4 °C. The pellet thus obtained was resuspended in 0.6 mL of phosphate-buffered saline, 0.1 mL of inhibitor solution was added, and the mixture was incubated for 10 min at 37 °C. Soft agar (2 mL) was then added for plating. The plates were incubated for 2 nights, the revertant colonies were counted, and the inhibitory activities were calculated.

Measurements of the Survival of the Bacteria. The titration of the bacteria was performed as described previously (Hayatsu et al., 1981). Thus, after treatment with a mutagen and an inhibitor, a 0.1 mL sample of the mixture containing bacteria, mutagen, and inhibitor was diluted 10^4-fold with phosphate-buffered saline, and then 0.1 mL of the diluted solution was mixed with soft agar (2 mL, containing histidine and biotin) for plating. The survival fraction (percent) is a value obtained as follows:

$$100 \times (\text{colonies in the presence of mutagen and beer}/\text{colonies in the presence of mutagen without beer}) \quad (2)$$

In all of the bacterial assays, duplicate plates were used for individual dose points and the averages of data from the two plates are shown.

Extraction of Hops. One and a half grams of hops was put into 1000 mL of boiling buffer of 10 mM sodium citrate at pH 5. After 90 min of boiling, the volume was adjusted to 1000 mL and the extract was filtered to remove the residual solids. This procedure was a simulation of the process of making beer; for beer production, hops (∼1.5 g) are put into a boiling solution (1000 mL) containing malt extracts at pH ∼5, and the mixture is boiled for 1−2 h (personal communication from Mr. S. Tada of Kirin Brewery Co.). Therefore, 1 mL of this hops extract corresponded to 1 mL of beer. Three hundred milliliters of the

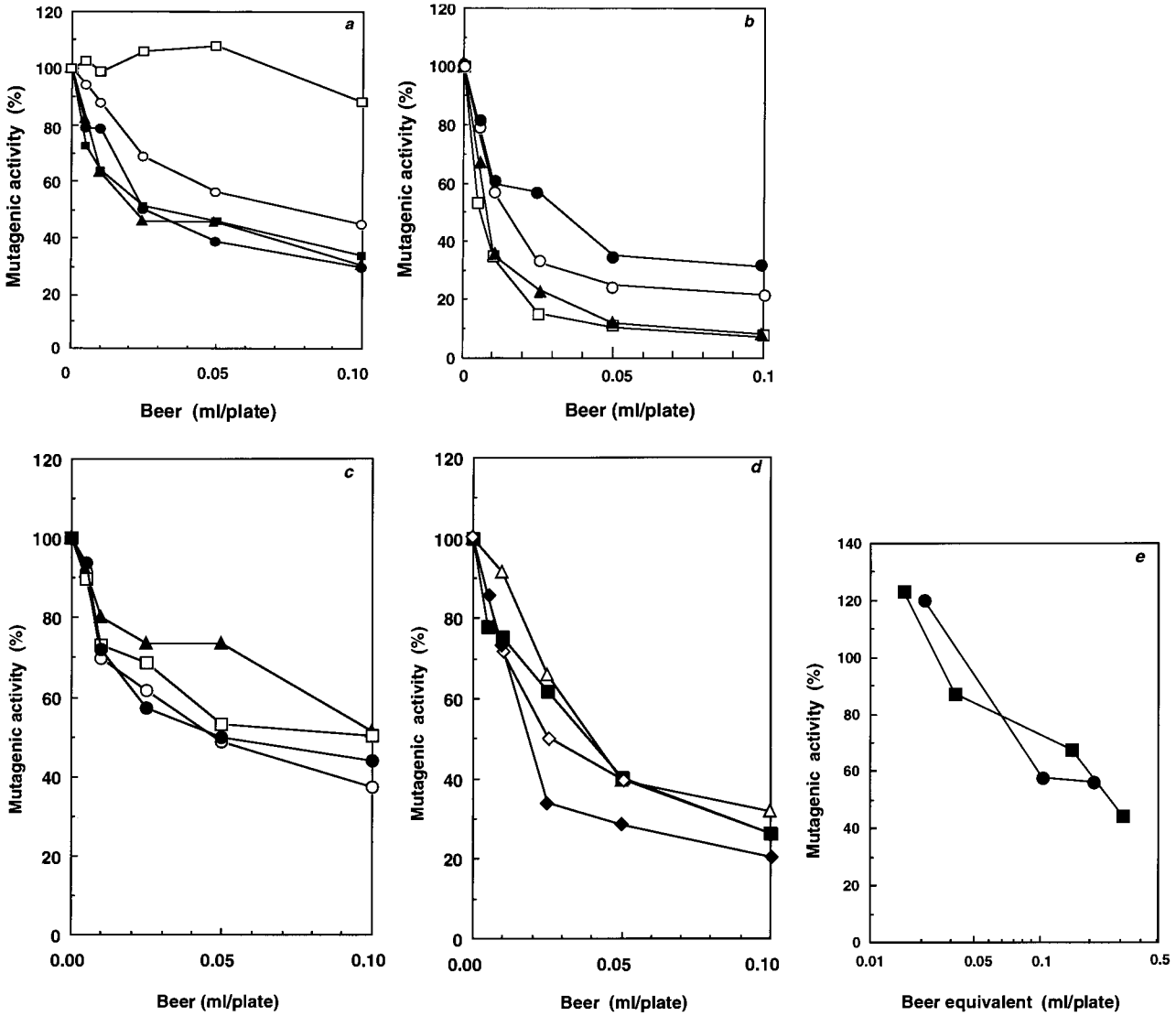

Figure 1. Effects of lager beer samples on the mutagenicity of Trp-P-2(NHOH) in *S. typhimurium* TA98: (a) effects of regular lager produced in Japan [lager A (●), lager B (○), lager C (■), lager D (▲), and lager E (□)]; (b) effects of black lager [lager F (□), lager G (▲), lager H (○), and lager I (●)]; (c and d) effects of lager beer produced outside Japan on the mutagenicity of Trp-P-2(NHOH) [(c) lager J (○), lager K (▲), lager L (□), and lager M (●); (d) lager N (△), lager O (◇), lager P (■), and lager Q (◆)]; (e) effects of freeze-dried and redissolved lager on the mutagenicity of Trp-P-2(NHOH) [lager A (●) and lager C (■)].

hops extract was freeze-dried, and the residue obtained (0.98 g) was dissolved in 14.7 mL of water. This solution was used for the antimutagenicity assays.

Analysis of Trp-P-2(NHOH) Decomposition. The decomposition of Trp-P-2(NHOH) mediated by plant phenols was monitored by absorption spectroscopy. The reaction was started by mixing an aqueous solution of Trp-P-2(NHOH) (final concentration = 2 μM) with a plant phenol in sodium phosphate buffer (0.08 M, pH 7.4) containing 0.08 M KCl. The absorption of 2 μM Trp-P-2(NHOH) at 260 nm was 0.11. The final concentration and the absorption at 260 nm of vanillic acid in the mixture were 1 μM and 0.064, those of ferulic acid 2.5 μM and 0.10, those of *p*-hydroxyphenethyl alcohol 250 μM and 0.10, and those of ellagic acid 0.25 μM and 0.10, respectively. The volume of the mixture was 2 mL. The reaction mixture was placed in a cuvette in a Hewlett-Packard 8450A UV−vis spectrophotometer with a constant mechanical stirring of the solution, and the spectra were recorded as a function of time, against a solution of respective plant phenol in the phosphate buffer as a reference.

Analysis of O^6-Methylguanine (O^6-meG) in DNA from *S. typhimurium* YG7108. An overnight culture of *S. typhimurium* YG7108 (50 mL) was mixed with MNNG (final concentration = 0.014 mM) and beer (stout A, 0−50 mL) in 0.07 M sodium phosphate (pH 7), in a total volume of 350 mL. The solution was incubated for 30 min at 37 °C with continuous mechanical shaking. The treated bacteria were then collected by centrifugation at 500 rpm for 10 min at 4 °C. The genomic DNA of *Salmonella* was prepared according to the method described in the literature (Wilson, 1997). The DNA obtained was dissolved in 0.1 N HCl (0.1 mL) and heated at 70 °C for 30 min. The mixture was then cooled on ice, and 2 volumes of ethanol was added to it. Precipitates were removed from the mixture by centrifugation, and the supernatant obtained was concentrated to dryness under a reduced pressure. The residue was dissolved in 0.05 mL of water and analyzed using HPLC on a Waters (Milford, MA) system coupled to a fluorescence detector (Arimoto-Kobayashi et al., 1997). The HPLC was performed with a column of ODS-80Ts (4.6 × 250 mm) purchased from Tosoh (Japan): column temperature, 40 °C; eluent, 0.1 M NH_4OAc (pH 5.0)/methanol (95:5); flow rate, 0.8 mL/min. O^6-meG was detected by fluorescence (287 nm excitation and 362 nm emission) and dG by the 247 nm absorbance.

Detection of DNA Adducts in Mice. Beer (stout A) was freeze-dried, and the solid obtained was dissolved in water of half the volume of original beer. Hereafter, this solution is called "×2 beer sol.". Two and four times water-diluted solutions of the ×2 beer sol. are called "×1 beer sol." and "×1/2 beer sol.", respectively. Five groups of animals, each composed

of three mice kept in a cage, were treated as follows. Starting from 3:00 p.m. on day 1, they were given, together with normal feed, plain water (group 1), the ×2 beer sol. (group 2), the ×1 beer sol. (group 3), the ×1/2 beer sol. (group 4), or the ×2 beer sol. (group 5), all ad libitum for drinking. After 72 h, 30 mg/kg of body weight Trp-P-2 was administered orally to each animal of groups 1−4 (∼0.2 mL of an aqueous solution of Trp-P-2). After an additional 24 h with access to the beer drinks, the animals were killed and their livers taken for analysis. One mouse was used as a no-treatment control, fed only normal diet with plain water and given no Trp-P-2. DNA was isolated from the liver by phenol extraction and analyzed according to the ^{32}P-postlabeling method using the intensification protocol described previously (Sugiyama et al., 1996). Briefly, the DNA was digested with micrococcal nuclease and spleen phosphodiesterase, and the digest was then labeled with ^{32}P under the adduct intensification conditions (Ochiai et al., 1993) by use of T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled mixture was further digested with nuclease P1 and phosphodiesterase I. The digest was subjected to ODS thin-layer chromatography (TLC) and contact-transferred to polyethyleneimine cellulose TLC (Randerath et al., 1984), and the plate was developed four times as described (Sugiyama et al., 1996). The radioactivities of adduct spots were analyzed with a Bio-Imaging analyzer (BAS 2000, Fuji Photo Film, Tokyo) with an exposure time of 2 h. The adduct level was determined as the relative adduct labeling (RAL) (Randerath et al., 1985), that is,

$$\text{RAL in the modified adduct intensification method} = (\text{count rate in adduct nucleotides}/\text{count rate in total nucleotides}) \times (\text{IF})^{-1} \quad (3)$$

where IF (intensification factor) is the value obtained in a Trp-P-2-only experiment, that is,

$$\text{IF} = \text{RAL in the modified adduct intensification method}/\text{RAL in the modified standard method} \quad (4)$$

RESULTS

We examined the antimutagenicity of 24 samples of beer toward Trp-P-2(NHOH) in the Ames *Salmonella* assay. Four samples of regular lager beer produced in Japan (lagers A−D) showed inhibition of Trp-P-2(NHOH) mutagenicity (Figure 1a). One sample (lager E) did not inhibit the activity. The amount of beer needed for 50% inhibition of Trp-P-2(NHOH) mutagenicity (I_{50}) was 0.05−0.1 mL. The survival fractions for Trp-P-2(NHOH) plus 0.1 mL of beer were 94.3% for lager A, 118.9% for lager B, 90.6% for lagerC, 100.1% for lager D, and 100.9% for lager E. Black domestic lager (lagers F−I) and lager from outside Japan (lagers J−Q) all inhibited the mutagenicity of Trp-P-2(NHOH) (Figure 1b−d). The I_{50} values for the black lager, 0.01−0.02 mL, were markedly lower than those for the regular lager (the I_{50} for the nondomestic lager beer was 0.02−0.1 mL) (Figure 1c,d).

Two beer samples (A and C) were freeze-dried, and the residues were tested for the inhibition of the mutagenicity. The results (Figure 1e) show that the efficacy of the inhibition was similar to that of the original beer. The bacterial survival fractions at the data point of Trp-P-2(NHOH) plus 0.1 mL of beer were 100−120% in all of these experiments. Two ale samples and four samples of stout beer were antimutagenic against Trp-P-2(NHOH), but the nonalcoholic beer showed no effect (Figure 2). The I_{50} of stout beer was the lowest among the beers tested, 0.005−0.01 mL, which corresponds to $\sim^1/_{10}$ of that for the lager. Again, there was no bacterial killing observed in these experiments. (These efficacies of the inhibitory action were similar among beer samples of the same type.) Japanese sake, brandy, and red and white wines were effective in inhibiting the mutagenicity of Trp-P-2(NHOH), but whiskey and shochu were not (Figure 3). The I_{50} was 0.01 mL for sake and red and white wines, 0.01−0.02 mL for brandy, and >0.1 mL for whiskey. Again, no appreciable killing of bacteria was noted. Beer also inhibited the activity of MNNG, Glu-P-1(NHOH), and activated Trp-P-1 (Figure 4a−c). For 50% inhibition of activated Trp-P-1, the dose for stout A was $\sim^1/_{10}$ of that for lager A, but the efficacies of the inhibitory action against MNNG and activated Glu-P-1 were similar for lagers A and B and stout A. Although the mutagenicity of BPDE was decreased only 20% by 0.1 mL of lager A or B, stout A was effective in inhibiting the mutagenicity (Figure 4d). Beer showed no inhibitory effects toward the mutagenicity of methylmethanesulfonate, IQ(N-HOH), activated IQ, activated PhIP, activated MeIQ, activated MeIQx, or activated 2-aminofluorene (data not shown). The survival in the presence of beer samples with these carcinogens was also examined, and we confirmed no decrease in survival in any of these experiments.

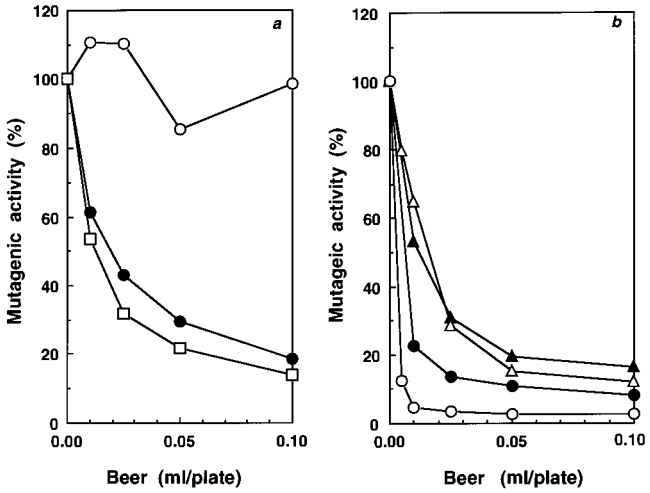

Figure 2. Effects of nonalcoholic beer, ale, and stout beer on the mutagenicity of Trp-P-2(NHOH) in *S. typhimurium* TA98: (a) nonalcoholic beer (○), ale A (□), and ale B (●); (b) stout A (●), stout B (○), stout C (▲), and stout D (△).

Stout A inhibited the O^6-meG formation by MNNG in the DNA of *S. typhimurium* YG7108 (Table 1). The decrease in O^6-meG was dependent on the dose of beer. The concentration of beer in this experiment, 50 mL of beer in 350 mL of the reaction mixture, corresponds to 0.1 mL of beer in 0.7 mL of the preincubation mixture of the Ames assay described in Figure 4a, in which the mutagenicity of MNNG was decreased to 16.4%. The decrease in the formation of O^6-meG to 16% by this beer concentration was consistent with the decreased mutagenicity. The possibility of any effects due to ethanol was eliminated by inclusion of ethanol at a fixed concentration (1.1%) in these treatments.

We examined the interference of stout A on the formation of DNA adducts by Trp-P-2 in vivo (Table 2). The adducts in the liver DNA of CDF_1 mice, given Trp-P-2 on day 3 in the course of a four-day continuous administration of freeze-dried beer (stout A), were measured using the ^{32}P-postlabeling method. The adducts detected were significantly decreased by the administration of 2× concentrated beer constituents, as compared with those in mice given Trp-P-2 without the beer.

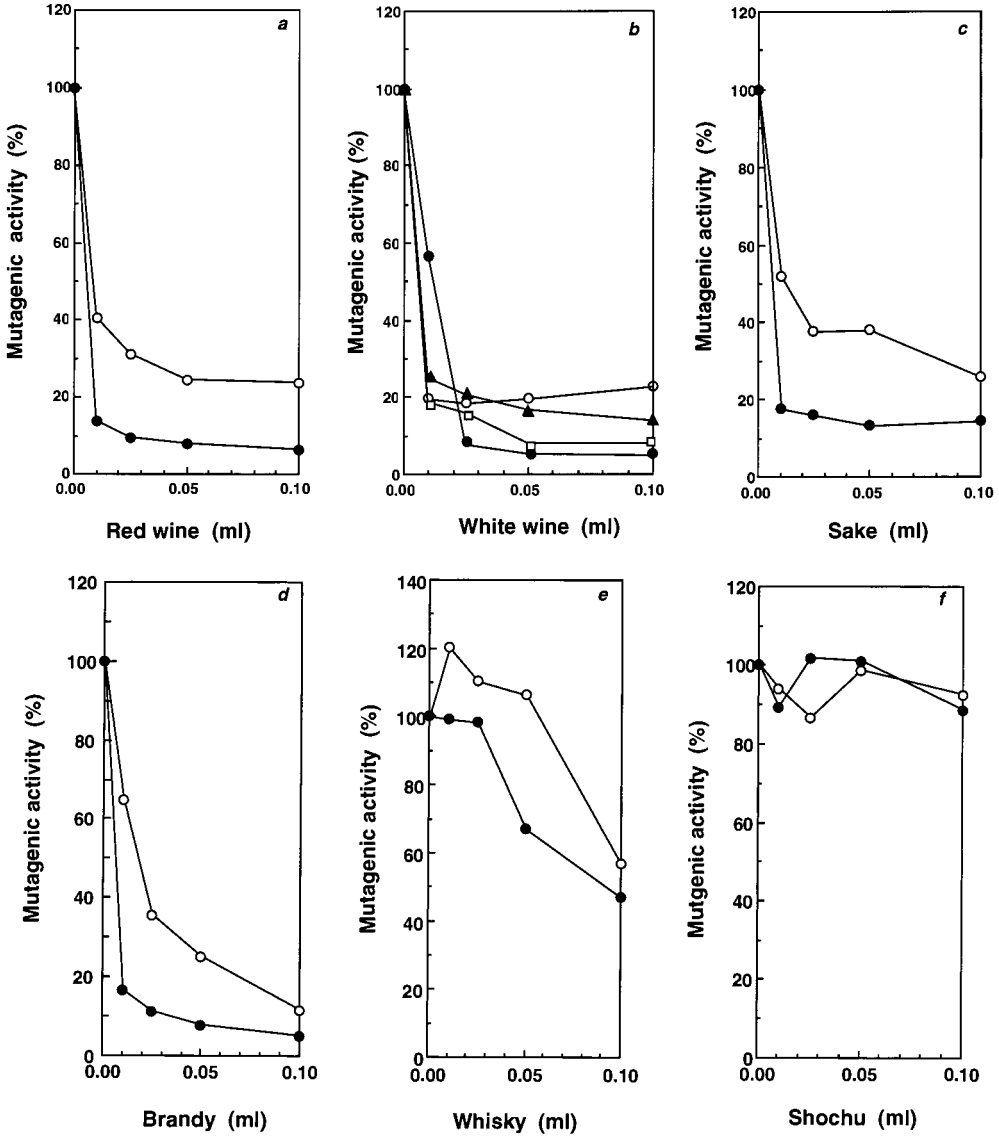

Figure 3. Effects of alcoholic beverages other than beer on the mutagenicity of Trp-P-2(NHOH) in *S. typhimurium* TA98: (a) effects of red wine [red wine A (●) and red wine B (○)]; (b) effects of white wine [white wine A (●), white wine B (○), white wine C (▲), and white wine D (□)]; (c) effects of sake [sake A (●) and sake B (○)]; (d) effects of brandy [brandy A (●) and brandy B (○)]; (e) effects of whiskey [whiskey A (●) and whiskey B (○)]; (f) effects of shochu [shochu A (●) and shochu B (○)].

To elucidate the inhibitory mechanisms of beer, we investigated the effect of a sequential treatment of bacteria with beer and the mutagen Trp-P-2(NHOH). Treatments of *S. typhimurium* with beer prior to or after the incubation with Trp-P-2(NHOH) resulted in no decrease of the mutant formation (Figure 5). It appears, therefore, that beer must be given to the bacteria together with the mutagen to exhibit its antimutagenic activity.

As beer is produced from hops, there is the possibility that the plant components originating from hops might be responsible for the antimutagenicity of beer. We found that a hot-water extract of hops inhibited the mutagenicity of Trp-P-2(NHOH) (Figure 6). We next examined the antimutagenicity of plant phenols known to be present in beer (Gramshaw, 1967): the chemical structures of the compounds tested are shown in Figure 7. Most of these plant phenols were found to inhibit the mutagenicity of Trp-P-2(NHOH) (Figure 8). For example, I_{50} of vanillic acid for Trp-P-2(NHOH) (20 pmol) was 5 μmol/plate and that of ferulic acid was 2 μmol/plate. Among the 12 compounds tested, protocatechuic acid and sinapic acid were not inhibitory. Except for *p*-hydroxyphenethyl alcohol, phloroglucinol, chlorogenic acid, and ellagic acid, these plant phenols inhibited the mutagenicity of MNNG (Figure 9). I_{50} of these plant phenols for MNNG (4 nmol) was 5−10 μmol/plate. We confirmed that there was no decrease in bacterial survival during these experiments. To examine the possibility that the plant phenols may affect the bacteria, we performed a sequential treatment of the bacteria with the phenol and Trp-P-2(NHOH). The mutagenicity of Trp-P-2(NHOH) was inhibited by a pretreatment of the bacteria with ellagic acid (Figure 10). However, no mutation inhibition was found in the treatment of bacteria with vanillic acid or ferulic acid, either prior to or after the incubation with Trp-P-2(NHOH).

To explore the mechanism of this plant phenol-mediated inhibition of Trp-P-2(NHOH) mutagenicity, we examined the effect of the phenols on the stability of Trp-P-2(NHOH). The decrease of absorbance at ~260 nm observable for an aqueous solution of Trp-P-2(NHOH) was accelerated by vanillic acid, *p*-hydroxyphenethyl alcohol, and ellagic acid and most markedly by ferulic acid (Figures 11 and 12).

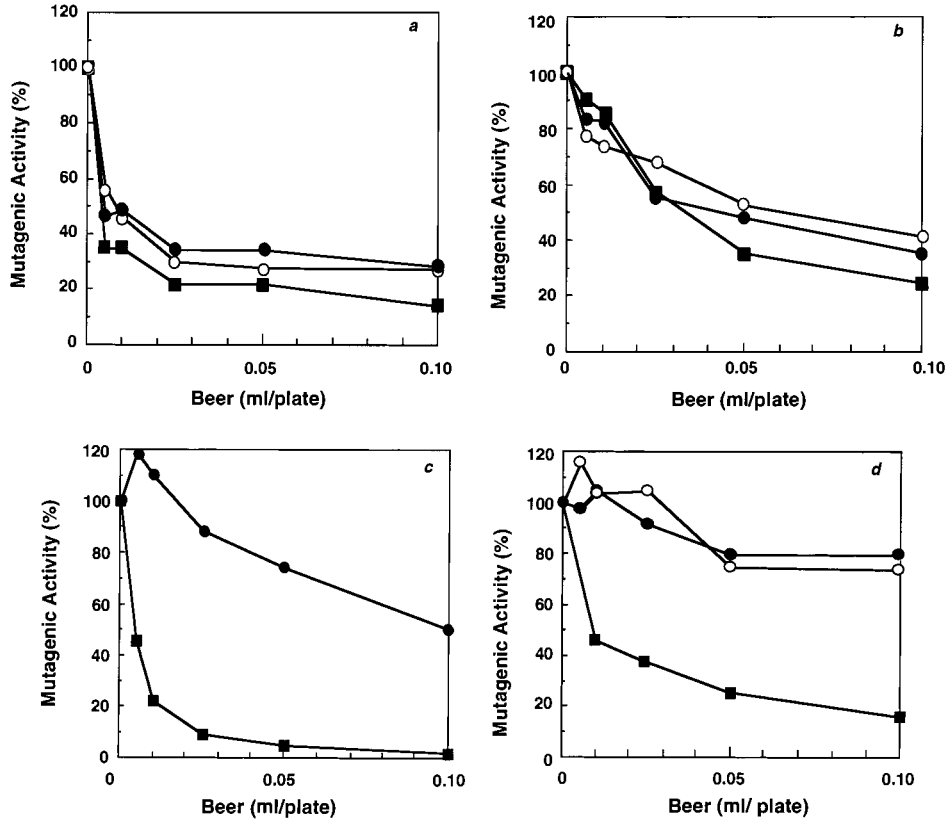

Figure 4. Effects of beer on the mutagenicity of MNNG in *S. typhimurium* TA100 (a), Glu-P-1(NHOH) in TA98 (b), activated Trp-P-1 in TA98 (c), and BPDE in TA100 (d): lager A (●), lager B (○), and stout A (■).

Table 1. Inhibitory Effects of Beer on the Formation of O^6-meG in *S. typhimurium* YG7108 Treated with MNNG

beer[a], mL	MNNG, μmol	O^6-meG/guanine ($\times 10^{-5}$)
0	5	5.83 (100%)
10	5	2.71 (46%)
50	5	0.945 (16%)
50	0	0[b]

[a] The total volume of the reaction mixture was 350 mL, which included 50 mL of bacterial suspension, which had an optical density at 600 nm of 0.8, and 250 mL of 0.1 M sodium phosphate, pH 7.4. Ethanol concentrations were fixed at 0.11% (v/v) in these experiments by supplementing appropriate amounts of ethanol to individual mixtures. [b] The detection limit was 0.5×10^{-5} for O^6-meG/guanine.

Table 2. Inhibitory Effects of Beer on the Occurrence of DNA Adducts of Trp-P-2 in Mice

group	beer sol.[a]	Trp-P-2, mg/kg of body wt	adducts/10^7 nucleotides	no. of mice
1	0	30	4.1 ± 0.26	3
2	×2	30	3.1 ± 0.21[c]	3
3	×1	30	3.3 ± 0.21	3
4	×1/2	30	2.9	3
5	×2	0	0[b]	3
no treatment	0	0	0	1

[a] Beer was freeze-dried and the residue dissolved in water of half the original volume. This solution was termed "×2 beer sol.". The two or four times water-diluted "×2 beer sol." were respectively termed "×1" and "×1/2 beer sol." [b] Detection limit was 1.0 adducts/10^7 nucleotides. [c] Significantly different ($p \leq 0.05$) from group 1 (*t* test).

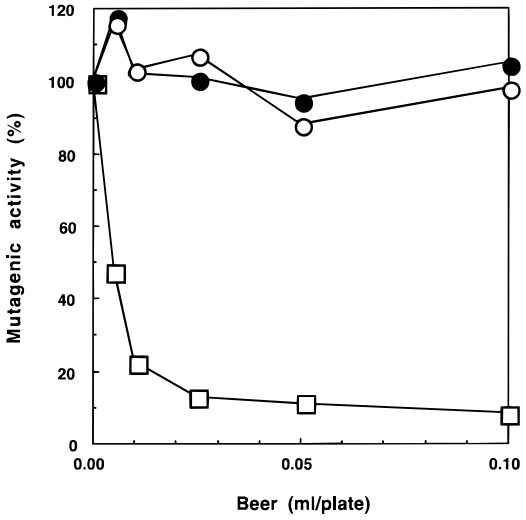

Figure 5. Effects of the sequential treatment of *S. typhimurium* TA98 with beer and Trp-P-2(NHOH). Pretreatment of bacteria with beer: bacteria were incubated with beer for 20 min at 37 °C, the beer was removed, and the bacteria were incubated with Trp-P-2(NHOH) for 10 min at 37 °C (●). Post-treatment of bacteria with beer: bacteria were incubated with Trp-P-2(NHOH) for 20 min at 37 °C, the Trp-P-2(NHOH) was removed, and the bacteria were incubated with beer for 10 min at 37 °C (○). The inhibitory effect of beer upon the mutagenicity of Trp-P-2(NHOH) was also determined by using the same methods as in Figure 1 (□).

DISCUSSION

The results described above show that beer and some other alcoholic beverages can inhibit the mutagenicity of Trp-P-2(NHOH), a proximate form of a mutagenic heterocyclic amine present in cooked food. As most beers that are produced both in Japan and in other countries showed antimutagenicity toward Trp-P-2(NHOH), the active compound(s) is (are) considered to be present in beer commonly. Because ethanol itself has no effect at its concentration in beer and because nonvolatile components of beer show antimutagenic effects (Figure 1e),

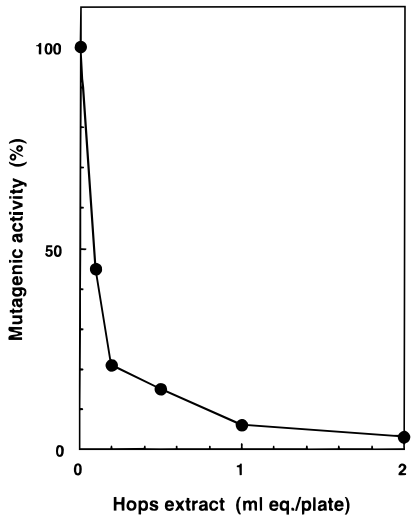

Figure 6. Inhibitory effect of a hops extract toward the mutagenicity of Trp-P-2(NHOH) in *S. typhimurium* TA98. "ml eq." represents the amount of hops extract equivalent to that in a corresponding volume of beer.

substances derived from the fermentation processes and/or raw materials of beer must be the cause of these antimutagenic effects. The 50% inhibition doses of stout beer against the mutagenicity of Trp-P-2(NHOH) and activated Trp-P-1 were $\sim 1/10$ of those of lager beer. In contrast, the I_{50} values against MNNG and Glu-P-1(NHOH) were similar between the stout and the lager. Lager beer is produced by use of the bottom yeast and the stout by use of the surface yeast, and the proportions of nonvolatile components are reported to be 3.3−4.4% for lager and 6.1% for stout (Resources Council, 1982). Possibly, there are several antimutagenic factors in the various beers, and the efficacy of them for the inhibition of the mutagenicity of individual mutagens may be different. The inhibitory effects of wine and sake were similar to that of stout beer. It is noteworthy that the protein content (0.2% in wine and 0.5% in sake), the carbohydrate content (2% in wine and 5% in sake), and the inorganic salt content (0.2−0.3% in wine) are comparable to those in stout (proteins, 0.6%; carbohydrates, 5.3%; inorganic salts, 0.2%) (Resources Council, 1982). There could be common antimutagenic factors present in beer and these beverages.

A distilled liquor, brandy, strongly inhibited the mutagenicity of Trp-P-2(NHOH) (Figure 4d), although whiskey and shochu, which are also distilled liquors, showed little or no inhibition (Figure 4e,f). Distilled liquors are supposed to consist of volatile components. The inhibitory factor(s) that must be present in these two brands of brandy is (are) expected to be different from those in beer and other fermented beverages.

Beer inhibited the MNNG-mediated methylation of guanine in the DNA of *S. typhimurium* (Table 1). It is likely that the inhibitory effect of the beer upon the mutagenicity of MNNG is a result of this suppression of DNA alkylation.

As the pre- and post-treatment of the bacteria with beer showed no antimutagenicity toward Trp-P-2(NHOH), it is unlikely that beer affects the bacterial system to reduce the membrane permeability toward Trp-P-2(NHOH) or to stimulate the repair of DNA modification caused by the mutagen. We consider that the antimutagenic substances in beer interact directly with the mutagens.

Because an extract of hops inhibited the mutagenicity of Trp-P-2(NHOH), the antimutagenic components in beer may be of plant origin. It has previously been hypothesized that polyphenolics in wine act as antioxidants in vivo (Soleas et al., 1997). Ellagic acid is present

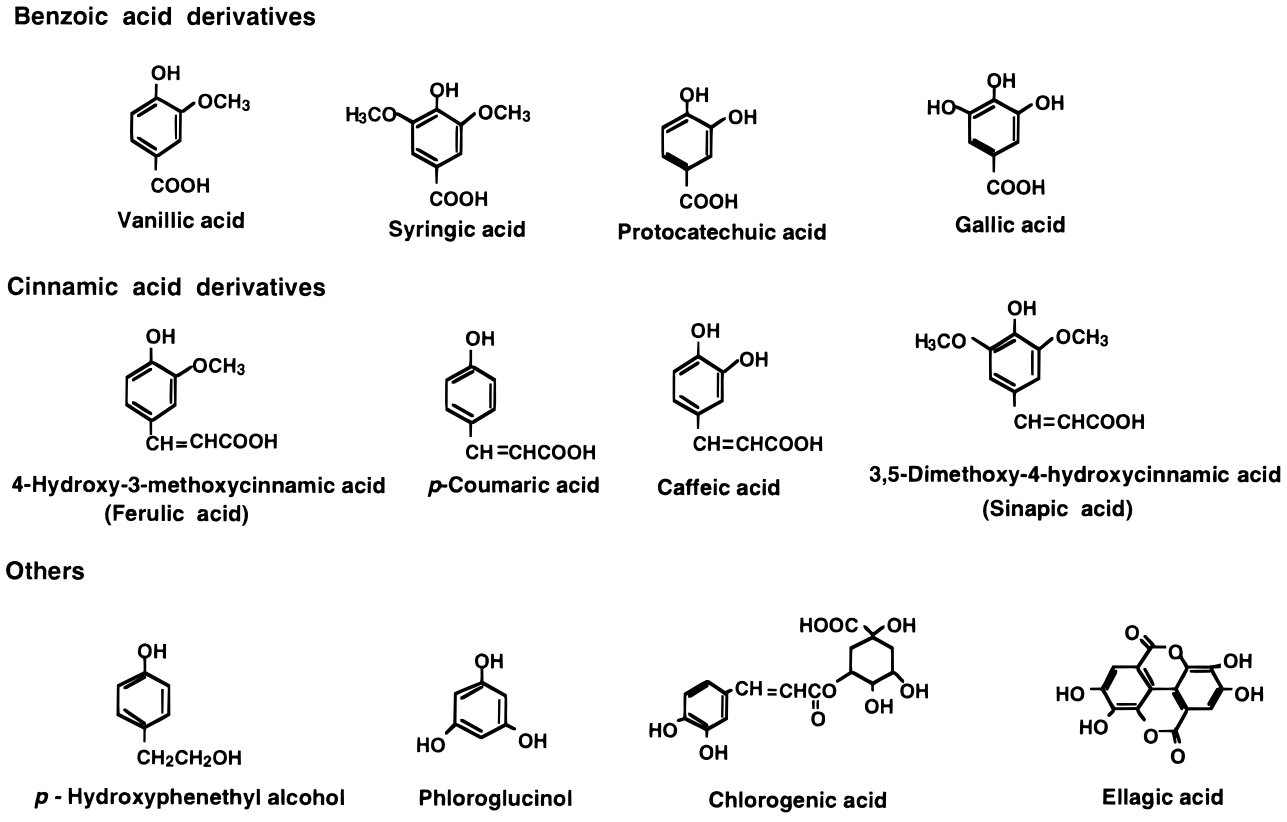

Figure 7. Structures of plant phenols contained in beer and wine.

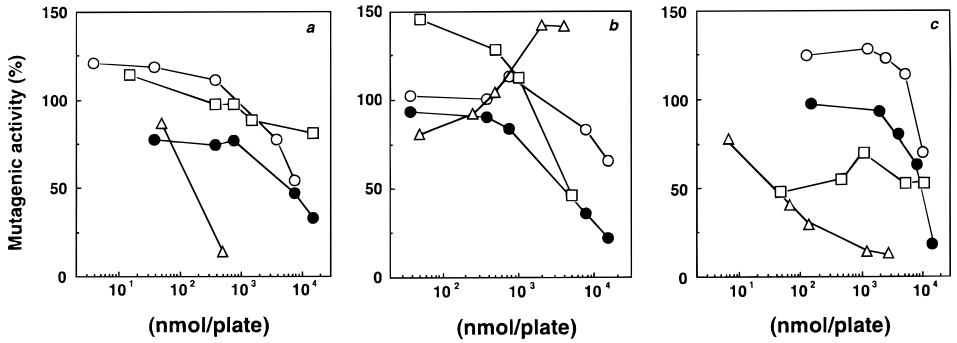

Figure 8. Inhibitory effect of the plant phenols toward the mutagenicity of Trp-P-2(NHOH) in *S. typhimurium* TA98: (a) benzoic acid derivatives [vanillic acid (●), syringic acid (○), protocatechuic acid (□), and gallic acid (△)]; (b) cinnamic acid derivatives [ferulic acid (●), *p*-coumaric acid (○), caffeic acid (□), and sinapic acid (△)]; (c) *p*-hydroxyphenethyl alcohol (●), phloroglucinol (○), chlorogenic acid (□), and ellagic acid (△).

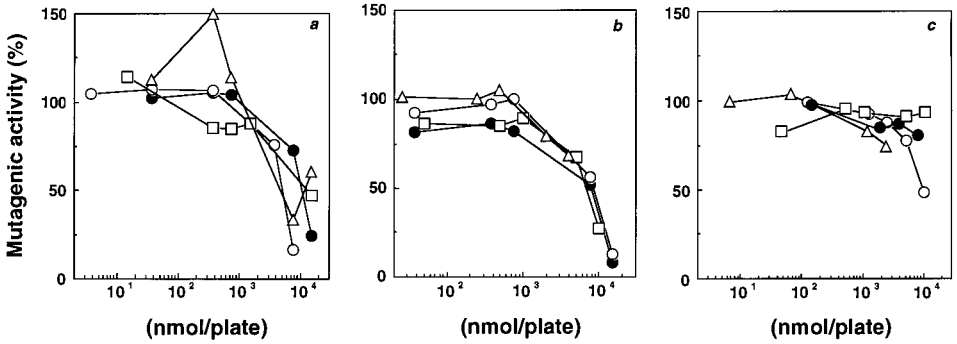

Figure 9. Inhibitory effect of the plant phenols toward the mutagenicity of MNNG in *S. typhimurium* TA100: (a) vanillic acid (●), syringic acid (○), protocatechuic acid (□), and gallic acid (△); (b) ferulic acid (●), *p*-coumaric acid (○), caffeic acid (□), and sinapic acid (△); (c) *p*-hydroxyphenethyl alcohol (●), phloroglucinol (○), chlorogenic acid (□), and ellagic acid (△).

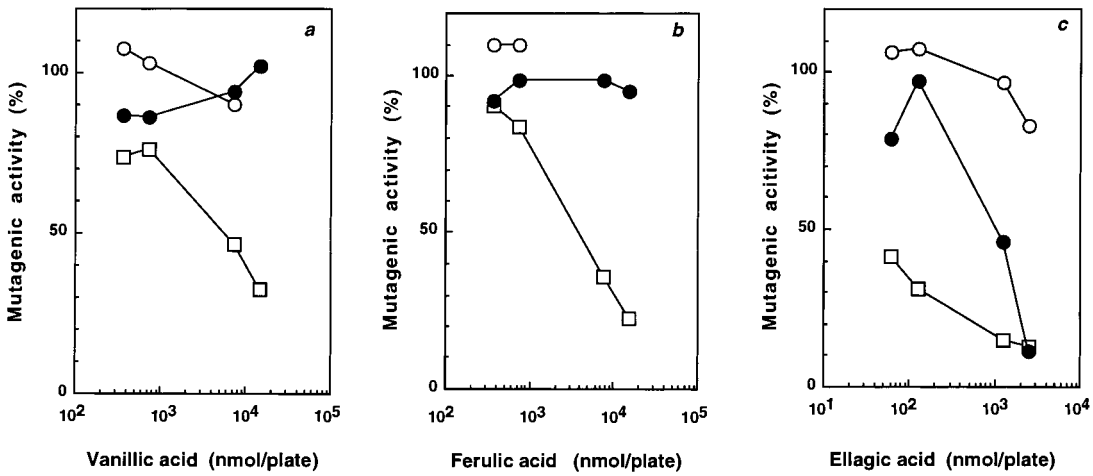

Figure 10. Effects of the sequential treatment of *S. typhimurium* TA98 with plant phenols and Trp-P-2(NHOH): (a) effect of vanillic acid [bacteria were treated with vanillic acid and then with the mutagen (●), with mutagen, and then with vanillic acid (○); simultaneous treatment with mutagen and vanillic acid (□)]; (b) effect of ferulic acid [ferulic acid and then mutagen (●); mutagen and then ferulic acid (○); simultaneous treatment with mutagen and ferulic acid (□)]; (c) effect of ellagic acid [ellagic acid and then mutagen (●); mutagen and then ellagic acid (○); simultaneous treatment with mutagen and ellagic acid (□)].

in grapes at high concentrations and is reported to be a potent inhibitor in vitro against BPDE (Wood et al., 1982) and *N*-methyl-*N*-nitrosourea (Dixit and Gold, 1986). We have shown here that several phenols in beer inhibit the mutagenicity of Trp-P-2(NHOH) and MNNG. Either a pre- or post-treatment of the *Salmonella* with vanillic acid or with ferulic acid resulted in no decrease in the mutagenicity of Trp-P-2(NHOH), and the same was also observed with beer. In contrast, a pretreatment of the *Salmonella* with ellagic acid resulted in a decrease in the mutagenicity of Trp-P-2(NHOH). Because ellagic acid is a wine component, but not a beer component, the antimutagenicity of wine could involve mechanisms distinct from those of beer.

Recently, we found that iron chlorophyllin mediates the conversion of Trp-P-2(NHOH) into its nitroso derivative (Arimoto-Kobayashi and Hayatsu, 1998). In that case, the spectrum changes were very rapid, being completed within a few minutes. Obviously, this kind of rapid chemical conversion is not operating in the antimutagenic actions of beer components: the spectrum changes are linear but not rapid (Figures 11 and 12). We showed previously that hemin, chlorophyllin, and *Monascus* pigments can suppress the Trp-P-2

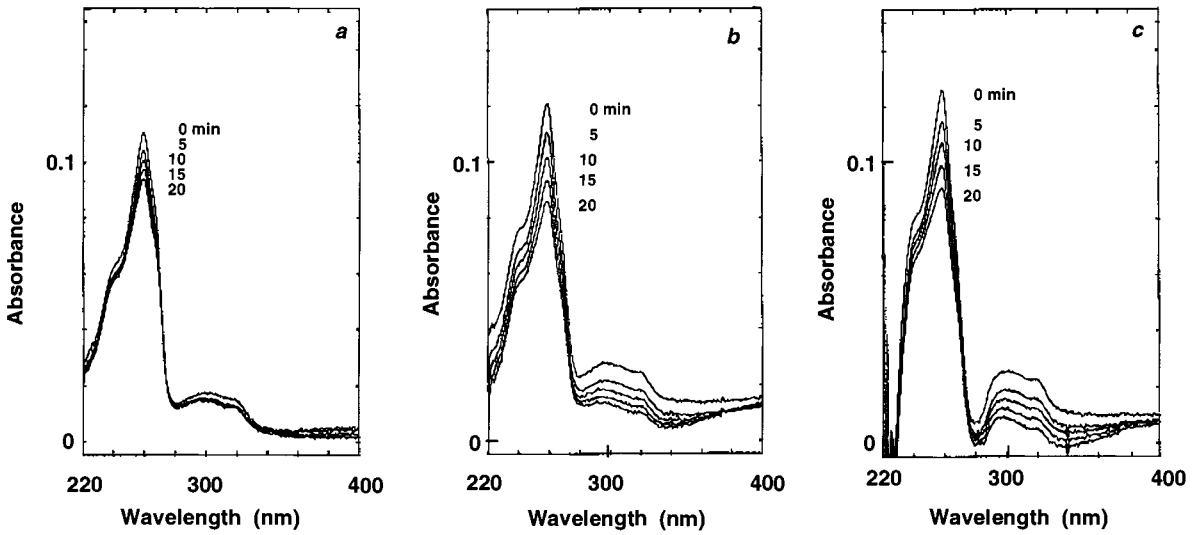

Figure 11. Changes of absorption spectra of Trp-P-2(NHOH) in the presence of plant phenol: (a) Trp-P-2(NHOH) alone (2 μM); (b) Trp-P-2(NHOH) (2 μM) plus vanillic acid (1 μM); (c) Trp-P-2(NHOH) (2 μM) plus *p*-hydroxyphenethyl alcohol (250 μM). These solutions contained 80 mM sodium phosphate buffer (pH 7.4) and 80 mM potassium chloride. The numbers given beside the spectra represent the time in minutes of incubation at which the spectra were recorded. The incubations were at room temperature (20 ± 5 °C).

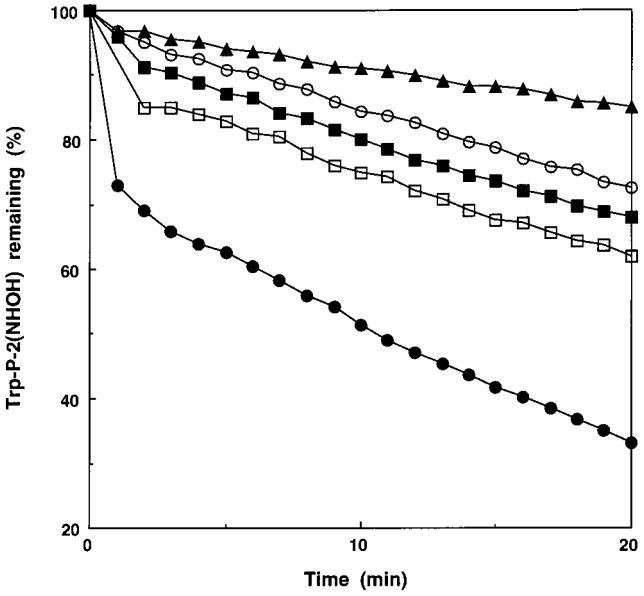

Figure 12. Time-dependent decrease in UV absorption of Trp-P-2(NHOH) at 260 nm. The UV absorptions were recorded at room temperature (20 ± 5 °C) every 60 s until 20 min: Trp-P-2(NHOH) alone (2 μM) (▲); Trp-P-2(NHOH) (2 μM) plus vanillic acid (1 μM) (■); Trp-P-2(NHOH) (2 μM) plus ferulic acid (2.5 μM) (●);Trp-P-2(NHOH) (2 μM) plus *p*-hydroxyphenethyl alcohol (250 μM) (○); Trp-P-2(NHOH) (2 μM) plus ellagic acid (0.25 μM) (□). These solutions contained 80 mM sodium phosphate buffer (pH 7.4) and 80 mM potassium chloride.

mutagenicity and can accelerate the degradation of Trp-P-2(NHOH) (Arimoto et al., 1980; Arimoto and Hayatsu, 1989; Izawa et al., 1997). Wood et al. (1982) reported that ellagic acid stimulates the decomposition of BPDE. It seems likely that the inhibition observed in the present work also arises by the enhanced degradation of the ultimate active metabolites.

The content of ferulic acid in beer is reported to be 15 mg/L and that of caffeic acid 5 mg/L (Herrmann and Mosel, 1973). The I_{50} of either ferulic acid or caffeic acid against the Trp-P-2(NHOH) was 1 mg, equivalent to 66 and 200 mL of beer, respectively. Therefore, these phenolics account only for a small part of the antimutagenicity of beer. One possibility is that the antimutagenicity of beer is a result of a cumulative effect of beer constituents which may individually have weak effects. The other possibility is that there are antimutagenic components other than those tested here. Because beer contains numerous components, we suspect that it would be difficult to pinpoint particular component(s) for the antimutagenic properties detected here.

We observed previously that direct-acting mutagenicity emerges in the serum of mice that have been administered Trp-P-2 intravenously and that this direct-acting mutagenicity persists for a period of 0.5−6 h after the administration (Aji et al., 1994). Because Trp-P-2 itself is an indirect mutagen, the direct activity detected in the serum has been ascribed to the presence of metabolically activated Trp-P-2. It is known that Trp-P-2(NHOH) is a primary metabolite of Trp-P-2 (Ishii et al., 1980). Our study here demonstrates that an administration of freeze-dried beer in drinking water inhibits the Trp-P-2−DNA adduct formation in the liver. It may be anticipated that the antimutagenic Trp-P-2(NHOH)-degrading component(s) of beer may work after having been absorbed though the digestive tract.

Because of the widespread consumption of beer in human populations, its potential protective effects against mutagens are worthy of further studies.

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