Fragrant 4-Hydroxy-3(2*H*)-furanones Swiftly Scavenge Nitrous Acid Causing Concomitant Formation of the Components with Relatively Weak Mutagenicity

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Reaction of fragrant 4-hydroxy-3(2*H*)-furanones including 2,5-dimethyl-4 hydroxy-3(2*H*)-furanone (DMHF) and 4-hydroxy-2-(or 5)-ethyl-5-(or 2)-methyl 3(2*H*)-furanone (HEMF) present in many processed foods with nitrous acid was studied. It was found that DMHF or HEMF swiftly reacted with nitrous acid at a molar ratio of 2 : 1 at pH 3 and 37℃. The products from the reaction of DMHF or HEMF with 4 equivalents of nitrous acid in chloroform were analyzed. Elemental analysis of the unpurified products showed that nitrogen atoms were introduced into the products which were found to be composed of multiple unstable furanone ring-opened compounds. The DNA-breaking and reducing activity of parent DMHF and HEMF were lowered by the nitrous acid treatment. The unpurified products were weakly mutagenic to a *Salmonella typhimurium* TA100 strain without metabolic activation. It was found that DMHF and HEMF effectively scavenged nitrous acid accompanied by concomitant loss of DNA-breaking activity and formation of compounds with relatively weak mutagenicity.

Key words—— 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), 4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (HEMF), nitrous acid, mutagenicity

INTRODUCTION

A variety of fragrant 4-hydroxy-3(2*H*)-furanones are known to be present in various foodstuffs.[1] 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF) called "furaneol" is produced by the Maillard reaction[2,3] in many processed foods, and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF) is found in many processed foods including soy sauce[4] (Fig. 1). The HEMF content is thought to be an index of the quality of soy sauce,[5] and the HEMF content of a brand of soy sauce has been estimated to be more than 40 mg/liter.[6] We have found that 4-hydroxy-3(2*H*)-furanones including DMHF and HEMF generate reactive oxygen species to induce DNA single strand breaks *in vitro* and induce micronucleated peripheral reticulocytes following oral administration.[6–11] The DNA-breaking activity of DHMF has been confirmed by Yamashita *et al.*[12]

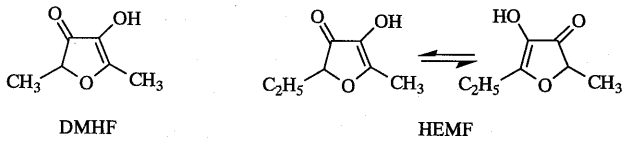

Fig. 1. Structures of DMHF and HEMF

Nitrate observed from the diet and secreted into saliva is converted into nitrite by bacteria in the oral cavity.[13,14] This may change into nitrous acid and serve as an agent to produce strongly mutagenic/carcinogenic compounds by reaction with their precursors under gastric conditions. Nitrosatable precursors of direct-acting mutagens have been found in foods.[15] It is important to investigate the food factors that regulate the action of nitrous acid. In the present study, the reaction profile of DMHF and HEMF with nitrous acid was studied to see whether the furanones scavenge or produce strongly mutagenic compounds. It was found that DMHF and HEMF effectively scavenged nitrous acid accompanied by concomitant loss of their DNA-breaking activ-

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ity and formation of compounds with relatively weak mutagenicity.

MATERIALS AND METHODS

Materials —— DMHF and HEMF were obtained from Tokyo Chemical Industry (Tokyo, Japan). Blue Rayon was purchased from Funakoshi Company (Tokyo, Japan). Supercoiled pBR 322 DNA (1.0 mg/ml in 10 mM Tris–HCl (pH 8.0) and 1 mM ethylenediaminetetraacetic acid (EDTA)) was obtained from New England Biolabs (Beverly, MA, U.S.A.).

Analysis —— Absorption spectra were obtained using a Hitachi U-2000 spectrophotometer (Tokyo, Japan). Nitrous acid was determined by the Saltzman method.[16] In this, an aliquot of the sample solution was made up to 25.0 ml with Saltzman solution composed of sulfanilic acid, *N*-(1-naphthyl)ethylenediamine and acetic acid, and the solution was kept at room temperature for 15 min. The absorbance of the solution at 545 nm was determined. The concentration of nitrous acid was determined by comparing the absorbance of 25.0 ml Saltzman solution containing 0.375 μmol sodium nitrite. Liquid chromatography/mass spectrometry (LC/MS) was performed using a Hitachi M-1000 LC API mass spectrometer (Tokyo, Japan). The test sample was dissolved into acetonitrile and chromatographed on a column of Inertsil ODS-3 (4.6 × 250 mm) (GL Sciences Inc., Tokyo, Japan). The column was eluted with a mobile phase composed of acetonitrile–water (1 : 1, v/v) at a flow rate of 0.5 ml/min. Ionization was carried out by atmospheric pressure chemical ionization.

Preparation of Nitrous Acid Solution in Chloroform —— A solution of 2 M sodium nitrite in water was adjusted to pH 3 by addition of concentrated hydrochloric acid, and the mixture was extracted with an equal volume of chloroform. The chloroform layer was dried over anhydrous sodium sulfate and the concentration of nitrous acid in the chloroform was estimated to be 250 mM by the Saltzman method.

Reaction of DMHF or HEMF with nitrous acid in chloroform —— A mixture of 10 mM DMHF or HEMF, and 40 mM nitrous acid in 100 ml chloroform was treated at 37℃ for 30 min. The solution was evaporated to dryness under reduced pressure, and a pale yellow paste was obtained. When a control solution, without DMHF and HEMF, was evaporated to dryness, the residue was found to be neutral and free from nitrite and chloride, indicating that nitrous acid and hydrogen chloride were removed by this work-up process.

Elemental analysis of 4 lots of paste derived from DMHF showed C, 47.97±0.46 (S.D.); H, 6.14±0.22; and N; 1.51±0.07%, the theoretical elemental composition of the parent compound of formula $C_6H_8O_3$ being C, 56.23; H, 6.29; and N, 0.00%. This result indicates that 0.16 of a nitrogen atom was introduced relative to 6 carbon atoms of the parent compound. Elemental analysis of 4 lots of paste derived from HEMF showed C, 50.34±0.33 (S.D.); H, 6.70±0.12; and N; 0.95±0.12%, the theoretical elemental composition of the parent compound of formula $C_7H_{10}O_3$ being C, 59.12; H, 7.09; and N, 0.00%. This result indicates that 0.11 of a nitrogen atom was introduced relative to 7 carbon atoms of the parent compound.

DNA Strand-Breaking Activity —— DNA strand-breaking activity was examined by the method described previously.[6–11] To 8 μl of 125 mM phosphate buffer (pH 7.4), were added 1 μl of supercoiled pBR 322 DNA solution at 100 μg/ml, and 1 μl of an aqueous solution of the paste obtained from the reaction of DMHF or HEMF with nitrous acid in chloroform. The mixture was incubated at 37℃ overnight. After addition of 1 μl of a mixture of 0.5% bromophenol blue/0.5% xylene cyanol/50% glycerol, the entire mixture was subjected to agarose gel electrophoresis run at 4 V/cm for 2 h using a Mupid-2 submarine electrophoretic apparatus (Advance Company, Tokyo, Japan). The buffer used for the electrophoresis contained 45 mM Tris–borate buffer (pH 8.3), 10 mM EDTANa_2, and 0.5 μg/ml ethidium bromide. The gel was prepared by dissolving 0.7% Sea-Kem ME agarose (FMC Bio-Products, Rockland, ME, U.S.A.) in buffer and the bands were visualized by irradiation at 300 nm.

Reduction of Fe(III) Ion —— Reduction of Fe (III) ion was monitored by the method of Emmerie and Engel.[17] A 5-ml solution of 1.5 mM $FeCl_3$ and the paste obtained by the reaction of DMHF with nitrous acid in chloroform (corresponding to 10—300 μM DMHF) in 10 mM Tris–HCl (pH 7.4) was incubated at 37℃ for 10 min. Then 1 ml of 0.5% dipyridyl in ethanol was added to the solution, and the mixture was finally made up to 25.0 ml. The absorbance of the solution at 520 nm was measured, and compared with that of a standard solution of $FeSO_4$.

Mutagenicity Assay —— The mutagenicity assay was carried out according to the preincubation method of Yahagi *et al.*[18] using *Salmonella typhimurium* TA 100 and TA98 strains,[19] with and without S9 mix. The microsomal S9 system prepared from the liver microsomes of a rat treated with phenobarbital

and 5,6-benzoflavone was obtained from Wako Pure Chemical Industries (Osaka, Japan). A 0.1-ml aliquot of a reaction mixture of DMHF or HEMF treated with aqueous nitrous acid, or a dimethyl sulfoxide (DMSO) solution of the paste obtained by reaction of DMHF or HEMF with nitrous acid in chloroform, was introduced onto the plate. Duplicate plates were used for the assay. The background numbers of His^+ revertant colonies/plate were 198—218 for TA100 without S9 mix, 198—205 for TA100 with S9 mix, 20—25 for TA98 without S9 mix, and 25—30 for TA98 with S9 mix. The number of His^+ revertant colonies was obtained after subtracting the background number of spontaneously formed His^+ revertant colonies/plate.

RESULTS

A solution of 1 mM nitrous acid was incubated with 0—2 mM DMHF, HEMF or ascorbic acid at pH 3 and 37℃ for 60 min under simulated gastric conditions. Nitrous acid was swiftly and completely destroyed by 2 mM DMHF (Fig. 2A) or HEMF (Fig. 2B) during 10 min, whereas the loss of nitrous acid produced by 2 mM ascorbic acid was moderate and incomplete (Fig. 2C). When 10 mM DMHF or HEMF was incubated with 0—40 mM nitrous acid at pH 3, the maximum absorption of these furanones at 286 nm was reduced in a dose-dependent manner for nitrous acid (Figs. 3A and B). The maximum absorption was completely abolished by incubation with nitrous acid above 10 mM. This result indicates that the furanone ring of these furanone derivatives was destroyed by nitrous acid treatment. Hence, DMHF or HEMF can swiftly react with nitrous acid at a molar ratio of 2 : 1.

When 10 mM DMHF or HEMF was treated with 40 mM nitrous acid (reactant molar ratio at 1 : 4) in chloroform at 37℃ for 30 min, the maximum absorption of these furanones at 286 nm was completely abolished (data not shown). The solution was evaporated to dryness under reduced pressure to remove nitrous acid, hydrochloric acid and chloroform, and a pale yellow paste was obtained. Elemental analysis of the unpurified paste obtained from the reaction of DMHF with nitrous acid in chloroform indicated that 0.16 of a nitrogen atom was introduced relative to 6 carbon atoms of DMHF. Elemental analysis of the unpurified paste derived from

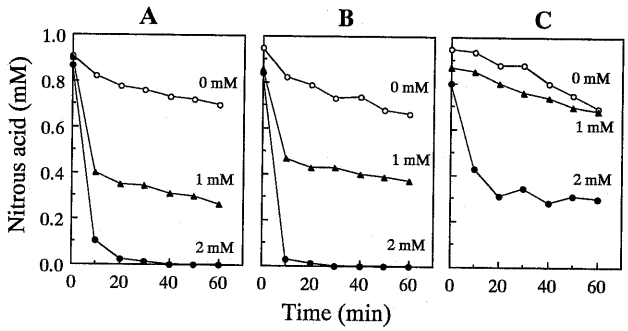


Fig. 2. Loss of Nitrous Acid by DMHF (A), HEMF (B) and Ascorbic Acid (C) at pH 3 and 37℃

A mixture of 1 mM sodium nitrite and DMHF, HEMF or ascorbic acid at the indicated concentration in water was adjusted to pH 3 by addition of concentrated hydrochloric acid, and incubated at 37℃ for the indicated period. The remaining nitrous acid was determined by the Saltzman method.

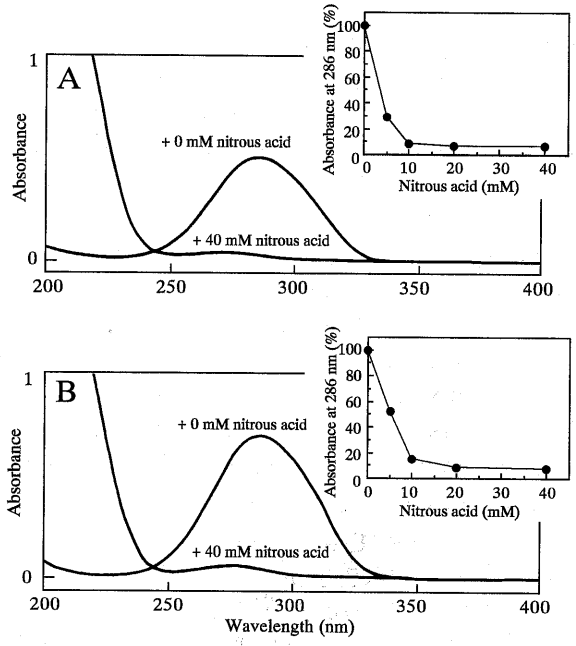


Fig. 3. Reduced UV Absorption of DMHF (A) and HEMF (B) Produced by Nitrous Acid at pH 3 and 37℃

A mixture of 10 mM DMHF or HEMF, and sodium nitrite at the indicated concentration in water was adjusted to pH 3 by addition of concentrated hydrochloric acid, and incubated at 37℃ for 30 min. After addition of 100-fold volume of water, the UV spectrum of the reaction mixture was measured. Inserts show the reduced absorbance of these furanones at 286 nm in a dose-dependent manner for nitrous acid. The absorption spectrum of the control mixture without nitrous acid remained unchanged during the incubation period.

HEMF indicated that 0.11 of a nitrogen atom was introduced relative to 7 carbon atoms of HEMF. The paste was analyzed by liquid chromatography/mass spectrometry (LC/MS) (Fig. 4), and the LC/MS peaks of the paste were monitored at various molecular weights (m/z) assuming the possible furanone ring-opened structures, dinitrite ester, mononitrite esters, chlorine-

substituted compounds, hydrolyzed compounds and oxidized compounds. The LC/MS patterns monitored at m/z 145, 147 and 205 are shown in Fig. 4A. These patterns showed that the products consisted of multiple components. The mass spectrum of the peak fraction at a retention time of 12.2 min (Fig. 4B) indicated a mass at m/z 205 ($M^+ + H$), corresponding to the dinitrite ester with a furanone ring-opened structure (molecular weight: 204) (I) (Fig. 5). The mass spectra of the peak fractions at retention times 10.8 and 3.7 min (Fig. 4B) indicated a mass at m/z 147 ($M^+ + H$), corresponding to the hydrolyzed forms of the nitrite ester (molecular weight: 146) (II), and a mass at 145 ($M^+ + H$), corresponding to the oxidized forms (molecular weight: 144) (III) (Fig. 5). Mass peaks corresponding to chlorine-containing components were not detected. Silica gel column chromatography of the paste obtained from DMHF using a solvent composed of chloroform–methanol (10 : 1, v/v) gave an iodine-positive fraction with an *Rf* value of 0.3 on thin-layer chromatography developed using the same solvent. Mass spectra of the fraction obtained using electron impact ionization, chemical ionization and fast atom bombardment ionization techniques suggested that the major components in the fraction were in the oxidized form (III). Several attempts to isolate and identify these products were made but were unsuccessful owing to the instability of the products.

DMHF[8] and HEMF[6] are able to cleave DNA single strands through generation of reactive oxygen species, and this activity may be assumed to be due to the reducing potency of the Fe(III) ion. In order to discover whether the DNA-breaking activity of these compounds was maintained following treatment with nitrous acid, the DNA-breaking activity of the paste obtained from the reaction of DMHF or HEMF with nitrous acid in chloroform was examined. The DNA-breaking activity was examined using a supercoiled plasmid DNA (form I) which is converted into a nicked open circular form (form II) by strand-breaking, and each form can be separated by agarose gel electrophoresis. While parent DMHF and HEMF were able to break DNA strands at more than 10 μM (Fig. 6A, lanes 2—4; B, lanes 2—3), no DNA-breaking activity of nitrous acid-treated DMHF at 10—100 μM was observed (Fig. 6A, lanes 5—7) and the DNA-breaking activity of nitrous acid-treated HEMF

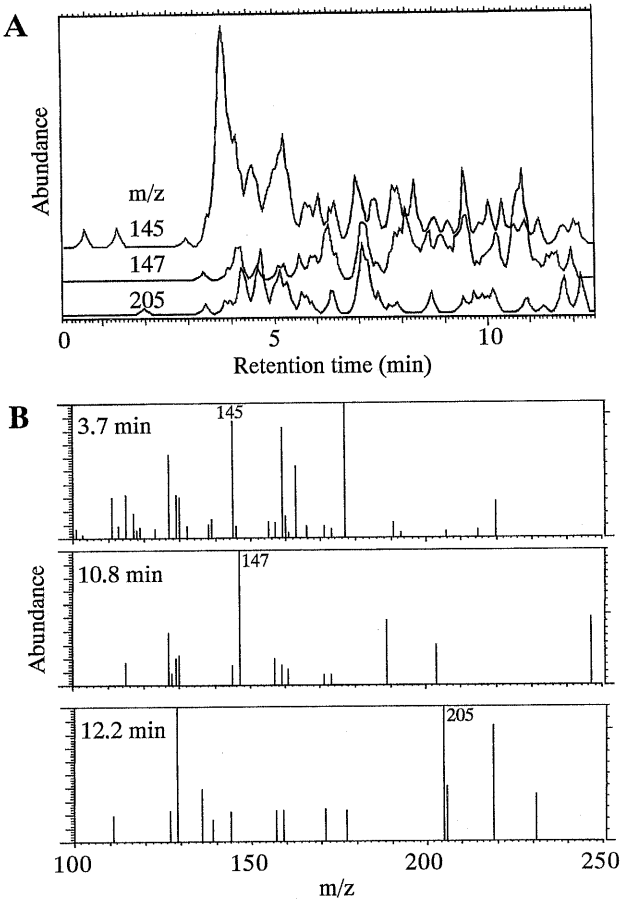
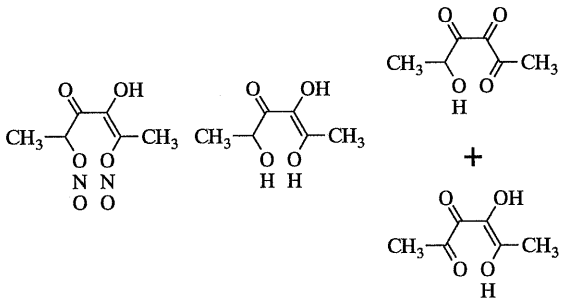


Fig. 4. LC/MS Pattern of the Paste Obtained following the Reaction of DMHF with Nitrous acid in Chloroform (A); and the Mass Spectra of the Peak Fractions at the Indicated Retention Times (B)



I (Molecular weight: 204) II (146) III (144)

Fig. 5. Possible Structures of the Reaction Products of DMHF with Nitrous Acid in Chloroform

at 10 μM was reduced (Fig. 6B, lanes 4—5). Hence, the DNA-breaking activity of the parent compounds was reduced by nitrous acid treatment. In order to know whether the reducing activity of parent DMHF to convert Fe(III) ion into Fe(II) ion[8] was maintained following treatment with nitrous acid, the reducing activity of the treated

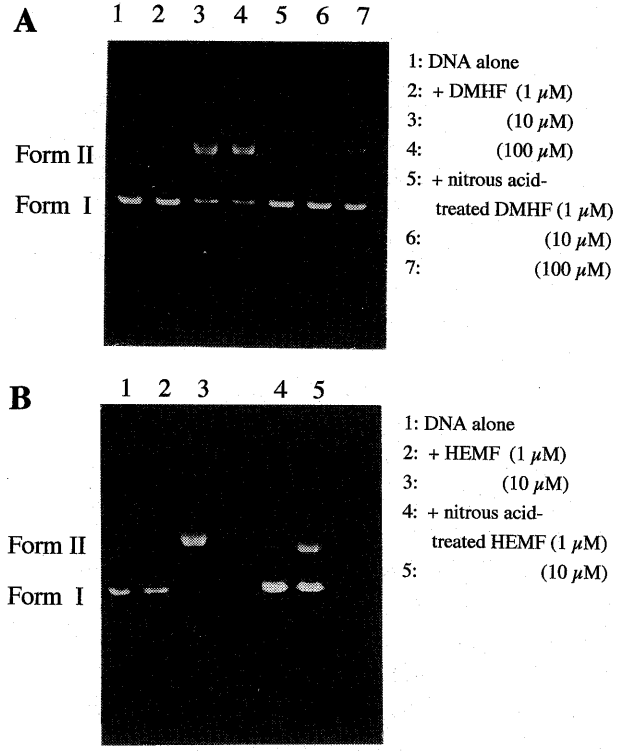


Fig. 6. Agarose Gel Electrophoresis of a Mixture of Supercoiled pBR 322 DNA and Nitrous Acid-Treated DMHF (A) and HEMF (B)

A mixture of pBR 322 DNA (10 μg/ml) and the paste obtained by the reaction of DMHF or HEMF with nitrous acid in chloroform in 0.1 M phosphate buffer (pH 7.4) was incubated at 37℃ overnight, and subjected to agarose gel electrophoresis. The electrophoretic positions of a supercoiled form (form I) and a nicked open circular form (form II) of DNA are indicated.

DMHF was examined. While the parent DMHF effectively reduced Fe(III) ions to Fe(II) ions, the activity of the treated DMHF was reduced (Table 1). The reduced DNA-breaking activity of DMHF through generation of reactive oxygen species may be due to the lowered reducing activity.

The mutagenicity of the reaction mixture of 100 mM DMHF or HEMF with 400 mM nitrous acid (reaction at a molar ratio of 1 : 4) incubated at pH 3 and 37℃ for 30 min was assayed using a *Salmonella typhimurium* TA100 strain without metabolic activation after stopping the reaction by addition of ammonium sulfamate. The mutagenicity of DMHF and HEMF was increased 6- and 14-fold, respectively, by this treatment (Figs. 7 A,B).

Table 1. Reducing Activity of Fe(III) Ion of Nitrous Acid-Treated DMHF

	Fe(II) ion formed (μM)	
Concentration (μM)	DMHF	Nitrous acid-treated DMHF
10	31	9
30	86	22
100	220	66
300	290	150

A mixture of 1.5 mM $FeCl_3$ and DMHF, or the paste obtained by the reaction of DMHF or HEMF with nitrous acid in chloroform, at the indicated concentration was incubated at pH 7.4 for 10 min. Fe(II) ion produced was determined by dipyridyl.

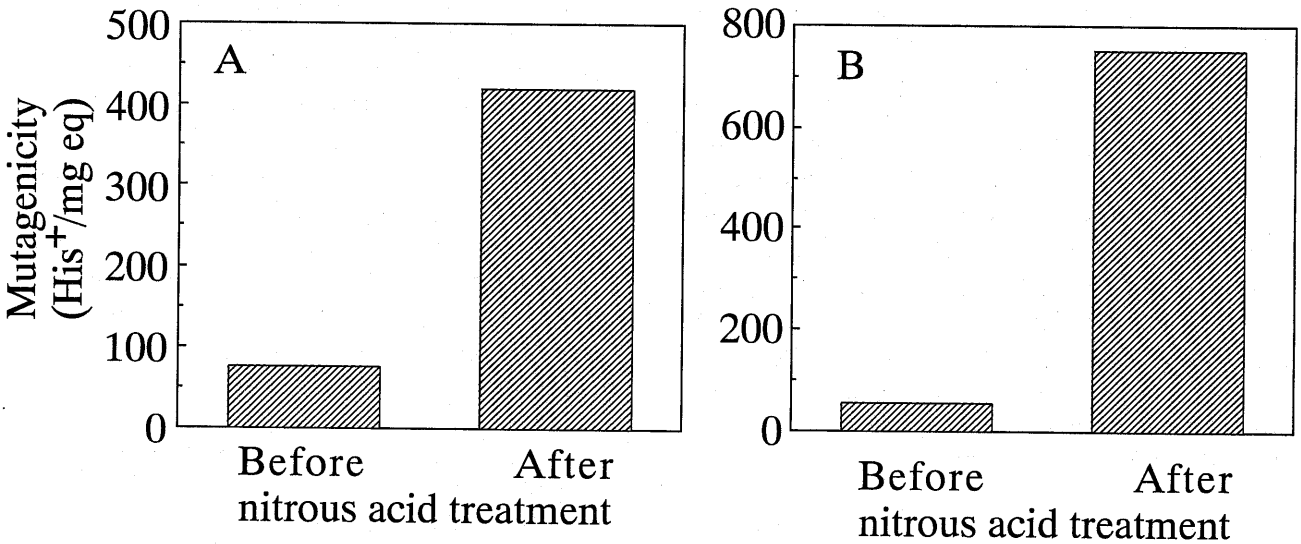


Fig. 7. Mutagenicity of DMHF (A) and HEMF (B) Treated with Nitrous Acid in an Aqueous Mixture at pH 3

A mixture of 100 mM DMHF or HEMF, and 400 mM sodium nitrite in water was adjusted to pH 3 by addition of concentrated hydrochloric acid and incubated at 37℃ for 30 min. The reaction was stopped by addition of 400 mM ammonium sulfamate. The mutagenicity of a 0.1-ml aliquot (corresponding to 2.5 μmol of the parent furanone, 318 μg for DMHF and 353 μg for HEMF) of the reaction mixture diluted 4-fold with water was tested on *Salmonella typhimurium* TA100 without metabolic activation.

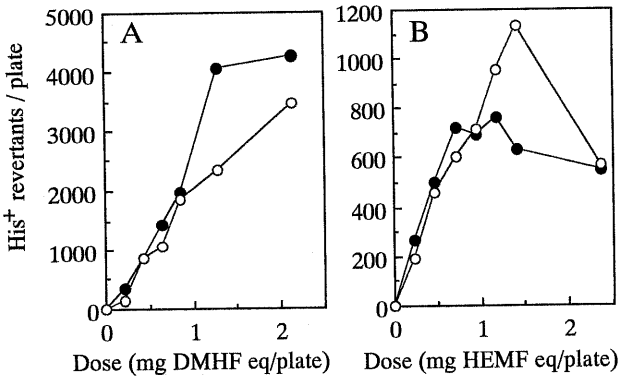


Fig. 8. Dose-Dependent Curves of the Mutagenicity of DMHF (A) and HEMF (B) Treated with Nitrous Acid in Chloroform

The paste obtained by the reaction of DMHF or HEMF with nitrous acid in chloroform was dissolved in DMSO. The mutagenicity of a 0.1-ml aliquot of the solution was tested on *Salmonella typhimurium* TA100 with (○) and without S9 mix (●).

The mutagenicity of the paste obtained by reaction of DMHF with nitrous acid in chloroform was examined using *Salmonella typhimurium* TA100 and TA98 strains, with and without metabolic activation (Figs. 8A, B). The mutagenicity of the products from DMHF to the TA100 strain without metabolic activation increased in a dose-dependent manner (Fig. 8A, closed circles). The number of His^+ revertant colonies was estimated to be 2000/mg DMHF equivalent. The mutagenicity was scarcely affected by the presence of S9 mix (Fig. 8A, open circles). The products were not mutagenic to the TA98 strain, with and without metabolic activation (data not shown). The mutagenicity of the products from HEMF to the TA100 strain without metabolic activation increased in a dose-dependent manner (Fig. 8B, closed circles). The number of His^+ revertant colonies was estimated to be 1000/mg HEMF equivalent. The mutagenicity was scarcely affected by the presence of S9 mix (Fig. 8B, open circles). The products were not mutagenic to the TA98 strain, with and without metabolic activation (data not shown). These results demonstrated that weak direct-acting mutagens were produced by reaction of DMHF or HEMF with nitrous acid.

DISCUSSION

A variety of fragrant 4-hydroxy-3(2*H*)-furanones and pyranone derivatives are known to be present in a wide variety of foods such as cognac, brandy, beer, coffee, soy sauce, cookies, sponge cake, cooked vegetables, cigarette smoke, beef, roasted sesame seeds, roasted almonds, strawberry jam, soybean paste, melon, lovage and cheese.[1] DMHF[2,3] and HEMF[4,5] are two representatives of these fragrant components. In particular, HEMF is present in a relatively high content in soy sauce,[6] and the quality of soy sauce has been evaluated with regard to the HEMF content.[5]

It was found in the present investigation that DMHF and HEMF swiftly reacted with nitrous acid at a molar ratio of 2 : 1 under simulated gastric conditions. These furanones were converted into unstable multiple furanone-ring-opened components by the reaction with nitrous acid in chloroform. The nitrous acid treatment caused a loss of DNA-breaking activity of parent DMHF and HEMF. DMHF[12] and HEMF[6] are able to cleave DNA single strands with the aid of metal ions through generation of reactive oxygen species. The loss of DNA-breaking activity may be caused by decreased reducing ability.

On treatment with nitrous acid, the mutagenicity of DMHF and HEMF to the *Salmonella typhimurium* TA100 strain without metabolic activation was increased. The number of His^+ revertant colonies of the nitrous acid-treated DMHF and HEMF was estimated to be 2000/mg DMHF equivalent and 1000/mg HEMF equivalent, respectively. However, the mutagenicity of nitrous acid-treated DMHF and HEMF was much lower than that of the nitrosated products of the known nitrosatable precursors.

The potent precursors already known are the condensates of tryptophan/acetaldehyde and tyramine in soy sauce, 4-chloro-6-methoxyindole in fava beans, indole-3-acetonitrile in Chinese cabbage, phenol and phenolics, sorbic acid, piperine and thiazolidines.[5] Methionine in fish can also be converted into a chlorine-containing direct-acting mutagen by treatment with nitrous acid/hydrochloric acid under gastric conditions.[20]

Wakabayashi *et al.*[21] have shown that condensates of tryptophan/acetaldehyde are one of the nitrosatable precursors in soy sauce. Their content in Japanese soy sauce is estimated to be 82—678 mg/liter, and the nitrosated products showed mutagenicity with 13000—17400 His^+ revertant colonies/mg to the *Salmonella*

typhimurium TA100 strain without metabolic activation. Ochiai *et al.*[22] have found another nitrosatable precursor, tyramine, in soy sauce. The tyramine content in soy sauce ranges from 20—2250 mg/liter, and the 3-diazotyramine produced was mutagenic with His^+ revertant colonies of 112000/mg to the TA100 strain without metabolic activation.

In conclusion, DMHF and HEMF can effectively scavenge nitrous acid under gastric conditions. Concomitantly, the DNA-breaking activity of DMHF and HEMF may be destroyed and weakly mutagenic components may be formed. The mutagenicity of nitrous acid-treated DMHF and HEMF may be much lower than known mutagenic nitrosated products.

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