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Identification of 4-Oxo-2-hexenal and Other Direct Mutagens Formed in Model Lipid Peroxidation Reactions as dGuo Adducts

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We searched for mutagens that react with 2′-deoxyguanosine (dGuo) in model systems of lipid peroxidation. To autoxidation systems of methyl linoleate (model of ω-6 fat), methyl α-linolenate (MLN) (model of ω-3 fat), and commercial salad oil, dGuo was added. The reaction mixtures were analyzed by HPLC. Six adducts were detected, and their structures were determined by ^{1}H and ^{13}C NMR, UV, and mass spectra and by comparison with synthetic authentic samples. The mutagens that reacted with dGuo to form these adducts were proposed as glyoxal, glyoxylic acid, ethylglyoxal, and 4-oxo-2-hexenal (4-OHE). The formation of 8-hydroxy-dGuo, an oxidized product of dGuo, was also detected in the model reaction mixtures. Among them, glyoxal and glyoxylic acid are known mutagens, while ethylglyoxal and 4-OHE, produced from MLN, have not been reported as mutagens thus far. We confirmed the mutagenic activity of 4-OHE with *Salmonella* strains, TA100 and TA104, without S9 mix. These compounds may be involved in lipid peroxide-related cancers.

Introduction

As diet is one of the main causes of human cancer, it is important to identify mutagens in food to prevent cancer. Epidemiological studies suggest that a high-fat diet is a risk factor for various cancers, such as breast and prostate cancer (*1*, *2*). On the other hand, it is known that an elevated risk of colon cancer is associated with red meat intake (*3*), while the role of total fat and specific fatty acids in colon carcinogenesis is not clear (*4*). Brink et al. suggested that a high-intake diet of polyunsaturated fatty acids is associated with an increased risk of mutated K-ras colon tumors, based on a cohort study (*5*). We have been interested in the mutagens produced by lipid peroxidation, because foods contain various polyunsaturated fatty acids and heme iron (Fe), a catalyzer of lipid peroxidation (*6*). Lipid peroxidation may occur during the cooking or storage of foods. After red meat ingestion, heme (ferroprotoporphyrin) is released by hemoglobin digestion and may enter the colon (*7*), while some of the ingested fat may also pass through the colon as triglycerides or as free fatty acids (*8*). Therefore, autoxidation systems of various unsaturated fatty acids with hemin are interesting models of lipid peroxidation reactions, to clarify the combined effect of a high-fat and red meat diet. Although aldehyde type mutagens, such as malondialdehyde, acrolein, crotonaldehyde, and 4-hydroxynonenal, have been identified (*9*−*11*), there have been no detailed studies on other mutagens, particularly those formed from ω-3 polyunsaturated fatty acids. In this report, we describe our identification of four mutagens, including new mutagens, as 2′-deoxyguanosine (dGuo)[1] adducts in model lipid peroxidation reaction mixtures containing hemin and methyl linoleate (MLA), methyl α-linolenate (MLN), and salad oil.

Experimental Procedures

Chemicals. dGuo and hemin were purchased from Sigma Chemical Co. (St. Louis, MO). MLA (purity > 99%) and MLN (purity > 99%) were obtained from Fluka (Buchs, Switzerland) and MP Biomedicals, LLC (Aurora, OH), respectively.

Reaction of dGuo with Products Formed in Lipid Peroxidation Model Systems and Analysis of the Adducts. dGuo (20 mg) and hemin (2.5 mg) were mixed in 50 mM phosphate buffer (pH 7.4) (10 mL) and were vigorously shaken with 1 mL of MLA, MLN, or commercial salad oil (rapeseed oil + soybean oil) in an open plastic tube (50 mL) to form a homogeneous emulsion. Occasionally, deionized water was added to maintain the volume of each reaction mixture. The reaction was continued for 3 days at room temperature. After centrifugation to separate the oil layer, 100 μL of the aqueous layer was injected into an HPLC column (CAPCELL PAK C18 MG, 5 μm, 4.6 mm × 250 mm, Shiseido Fine Chemicals, Japan) connected with a photodiode array UV detector (Hewlett-Packard 1100 HPLC Detection System). Used were the following linear gradients of acetonitrile concentrations in 0.1% acetic acid: 0−15 min, linear gradient of acetonitrile (0−5%); 15−28 min, linear gradient of acetonitrile (5−20%). Adduct samples for structure determination were isolated by repeated (about 50 times) rounds of HPLC, by injecting 1 mL of the reaction mixture into a wider column (10 mm × 250 mm).

Spectra Measurements. The mass spectrum (EI) of synthetic 4-OHE was measured on a JEOL JMS-BU20 spectrometer. The mass spectra (FAB) of the adducts were recorded with a JEOL

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[1] Abbreviations: dGuo, 2′-deoxyguanosine; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MLA, methyl linoleate; MLN, methyl α-linolenate; 4-OHE, 4-oxo-2-hexenal; 4-ONE, 4-oxo-2-nonenal.

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JMS-DX303 spectrometer and a JEOL JMS-T100LC. ^1H and ^{13}C NMR spectra were measured with JEOL JNM-A500 and JEOL JNM-ECA600 spectrometers, using DMSO-d_6 as the solvent and TMS as an internal reference.

X-ray Crystallography. X-ray crystallographic measurements were performed on a Rigaku AFC7R diffractometer, with graphite monochromated Cu Kα radiation and a rotating anode generator. Calculations of the measurements were performed using the teXan crystallographic software package (Molecular Structure Corporation).

Preparation of 10-Hydroxyl-11-oxo-1,N2-ethano-dGuo. dGuo (27 mg, 0.1 mmol) and glyoxylic acid monohydrate (9 mg, 0.1 mmol) were dissolved in 1 mL of DMSO and incubated at 45 °C for 4 days. The reaction mixture was diluted 10-fold with water, and the major product was purified by repeated rounds of HPLC (CAPCELL PAK C18, 10 mm × 250 mm; elution, 4% acetonitrile in water), to yield 8.8 mg (27%) of the objective compound.

Synthesis of (*E*)-4-Oxo-2-hexenal(4-OHE). (*E*)-4-Hydroxy-2-hexenal diethylacetal (19.7 g, 104 mmol), prepared by the method of Esterbauer and Weger (*12*), was dissolved in dry dichloromethane (500 mL), and then, activated MnO_2 (240 g, Aldrich) was added to the solution. After a 72 h incubation at room temperature, the reaction mixture was filtered, washed with dichloromethane (500 mL), and concentrated under reduced pressure. The residue was fractionated by silica gel column chromatograpy [column volume, 450 mL; elution, first with hexane and then with hexane:ethyl acetate (9:1, v/v)] to yield 13.9 g (71%) of the pale yellow product of 4-OHE diethylacetal. It was dissolved in a mixture of 70 mL of 1% citric acid and 30 mL of methanol and was stirred for 24 h at room temperature. The methanol was evaporated under reduced pressure, and then, the solution was saturated with NaCl and extracted with ether. The ether layer was washed with a saturated NaCl solution and dried with anhydrous $MgSO_4$. The ether layer was concentrated under reduced pressure to obtain 5.7 g (68%) of 4-OHE. Mass (EI), *m/z* 112. NMR (δCDCl$_3$, *J*) 9.79 ppm (d, 6.9 Hz), 6.90 ppm (d, 16.2 Hz), 6.79 ppm (dd, 6.9, 16.2 Hz), 2.74 ppm (q, 7.2 Hz), 1.17 ppm (t, 7.2 Hz).

Preparation of 10-(2-Oxobutyl)-1,N2-etheno-dGuo. dGuo (40.9 mg, 0.15 mmol) and 4-OHE (25.8 mg, 0.23 mmol) were dissolved in 8.2 mL of 50 mM sodium phosphate buffer (pH 7.4) containing 10% ethanol and incubated for 5 days at room temperature. The major product was purified by repeated rounds of HPLC (CAPCELL PAK C18, 10 mm × 250 mm; elution, 10% acetonitrile in water) to yield 28.8 mg (63%) of the objective compound.

Preparation of 9-Ethyl-10-(2-oxobutyl)-1,N2-ethenoguanine. 9-Ethylguanine (30 mg, 0.17 mmol) and 4-OHE (28.2 mg, 0.25 mmol) were dissolved in 8.2 mL of 50 mM sodium phosphate buffer (pH 7.4) containing 10% ethanol and incubated for 5 days at room temperature. The major product was purified by repeated rounds of HPLC (the same conditions as above) to yield 31.5 mg (57%) of the objective compound. Mass spectrum (FAB), $(M - H)^-$, *m/z* 272.1145 (272.1148, calcd for $C_{13}H_{14}N_5O_2$).

X-ray Crystallographic Analysis of 9-Ethyl-10-(2-oxobutyl)-1,N2-ethenoguanine (7). A colorless plate crystal, with approximate dimensions of 0.02 mm × 0.13 mm × 0.40 mm, was chosen for X-ray crystallography. The crystal data are as follows: empirical formula, $C_{13}H_{15}N_5O_2$; crystal system, monoclinic; lattice parameters, a = 13.996(1) Å, b = 7.401(1) Å, c = 14.643(1) Å, β = 114.756(6)°, V = 1377.4(3) Å^3; Z value, 4; D_{calcd}, 1.32 g/cm^3. μ (Cu Kα): 7.71 cm^{-1}. Of the 4914 reflections that were collected, 2352 were unique. The intensities of three representative reflections were measured after every 150 reflections. No decay correction was applied. The structure was solved by direct methods (SIR92) (*13*) and was expanded using Fourier techniques (DIRDIF94) (*14*). Nonhydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full matrix least-squares refinement was based on 2047 observed reflections and 181 variable parameters and was converged with unweighted and weighted agreement factors of R = 0.082 and Rw = 0.159. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.34 and −0.17e$^-$/Å^3, respectively.

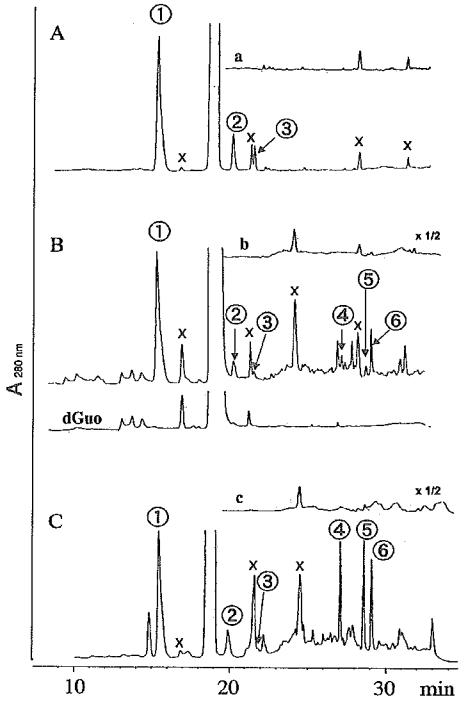


Figure 1. HPLC analyses of dGuo adducts formed by model lipid peroxidation reactions. (A) dGuo + MLA + hemin, (a) control reaction of MLA + hemin; (B) dGuo + salad oil + hemin, (b) control reaction of salad oil + hemin (attenuation, 1/2), (dGuo) control reaction of dGuo; (C) dGuo + MLN + hemin, (c) control reaction of MLN + hemin (attenuation, 1/2). The peaks labeled with x were also detected in the control reaction mixtures.

Mutagenicity Test. The bacterial mutagenicity test was carried out according to the method of Maron and Ames (*15*). In our experiments, we used a longer preincubation time, 60 min, and the plates were sealed with paraffin film (Parafilm, American National Can, United States) to prevent the evaporation of 4-OHE.

Results

Identification of Mutagens as dGuo Adducts Formed in Lipid Peroxidation Model Reactions. The method generally used to identify lipid peroxide-derived mutagens is to isolate them by HPLC, based on their mutagenic activity, before their structure determination. However, in the initial studies, we were not able to isolate unstable lipid peroxide-derived mutagens by HPLC. An alternative method to identify unstable mutagens is to trap them as stable dGuo adducts, because many mutagens and carcinogens react with DNA and nucleosides, particularly dGuo (*16*).

Using this method, the products formed in the model lipid peroxidation reaction mixtures (MLA + hemin, MLN + hemin, and salad oil + hemin), which are models of high-fat and red meat diets, were reacted with dGuo by vigorous shaking to form a homogeneous emulsion at a physiological pH (7.4). The reaction mixture was fractionated by HPLC coupled to a photodiode array UV detector. Six dGuo adducts, which were not produced from the control reaction mixtures of dGuo only or lipid peroxidation only (−dGuo), were detected by HPLC, as shown in Figure 1.

Adducts 1 and 2 were produced in both the MLA and the MLN reactions (Figure 1), while adducts 4−6 were detected in the MLN reaction mixture (Figure 1C). The amounts and the numbers of different adducts were higher in the MLN reaction mixture (Figure 1C) than the MLA reaction mixture (Figure

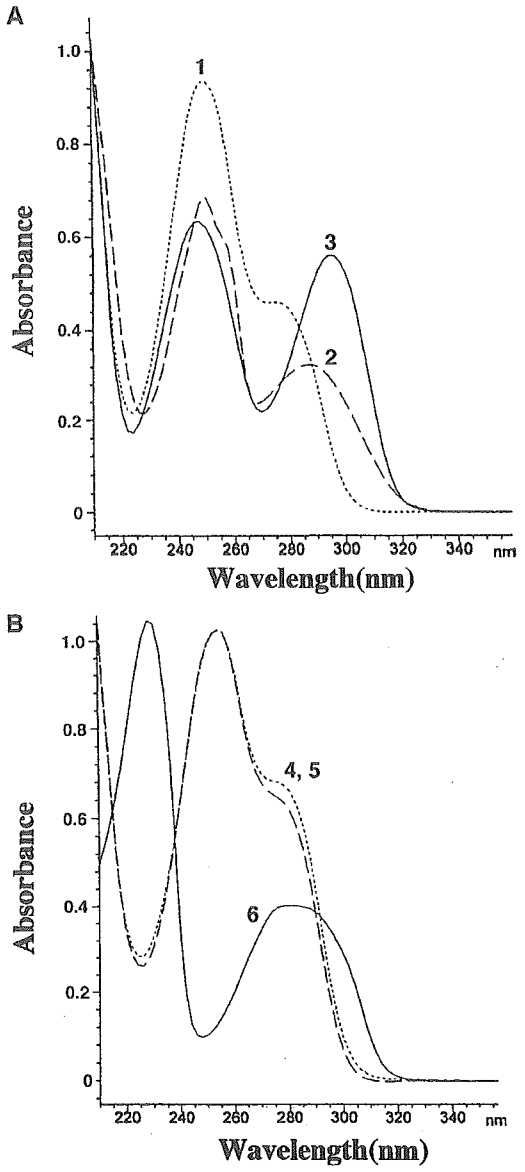


Figure 2. UV spectra of dGuo adducts. (A) Adducts 1 (• • •), 2 (- - -), and 3 (—); (B) adducts 4 (- - -), 5 (• • •), and 6 (—).

1A). Adduct 3 (8-OH-dGuo) was produced with a higher yield in the MLA reaction (Figure 1A) than in the MLN reaction (Figure 1C). The salad oil reaction generated an HPLC profile (Figure 1B) including some adducts also generated in the MLA (Figure 1A) and MLN reactions (Figure 1C).

Structure Determinations of Adducts. Adduct 1 was easily identified as glyoxal-dGuo (Figure 3, structure **1**), based on its characteristic UV spectrum (λ_{max}, 249 nm, shoulder at 275 nm) (Figure 2A-1). This identification was confirmed by a comparison with the synthetic compound prepared from glyoxal and dGuo (*18*–*20*).

The mass spectrum, $(M - H)^-$, *m/z* 322.0787 (322.0788, calcd for $C_{12}H_{12}N_5O_6$) of adduct 2 showed an increase of 56 mass units (C_2O_2) from dGuo. One of the possible structures was a cyclic adduct formed from glyoxylic acid and dGuo. Thus, compound **2** was prepared as a major reaction product of glyoxylic acid and dGuo. The retention time in HPLC and the ^{1}H NMR, UV, and mass spectral data of adduct 2 were identical to those of the synthetic **2**. The ^{13}C and ^{1}H NMR data of

Table 1. ^{13}C and ^{1}H NMR Assignments for Compound 2[a]

	δC (ppm)	δH (ppm)	multiplicity	*J* (Hz)
1N				
2	151.47			
3N				
4	147.88, 147.91			
5	120.32, 120.38			
6	154.24			
7N				
8	136.67, 136.70	8.14	s	
9N				
10	77.61	5.71	d	9.0
		5.72	d	9.0
11	171.25			
12N				
1′	83.12	6.17	dd	6.4, 6.0
2′	39.84	2.27	dddd	13.4, 6.0, 3.0, 3.0
		2.55	br. dd	13.4, 6.4
3′	70.55	4.35	m	
4′	87.84	3.83	m	
5′	61.51	3.52	m	
10 OH		7.81	d	9.0
		7.83	d	9.0
3′OH		5.31	br d	
5′OH		4.92	br t	

[a] 1′–5′, numbering of sugar carbons.

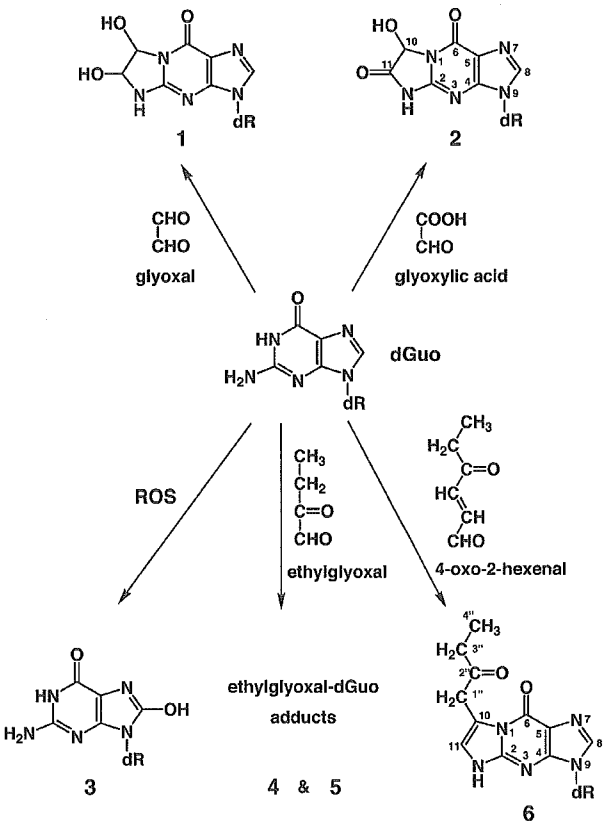


Figure 3. Structures of dGuo adducts formed by model lipid peroxidation reactions and their causative mutagens.

synthetic **2** are shown in Table 1. The proposed structure **2** was confirmed by ^{1}H–^{1}H two-dimensional (2D) correlation spectroscopy (COSY) and ^{1}H–^{13}C 2D heteronuclear multiple-bond correlation (HMBC) NMR studies using synthetic **2** (Figures 4 and 5). The three-bond correlation between H-10, C-10, N-1, and C-6 observed in the HMBC spectrum (Figure 5) clearly shows that adduct 2 is an 11-oxo-10-hydroxy compound, and the possibility of the regioisomer, the 11-hydroxy-10-oxo compound, should be ruled out.

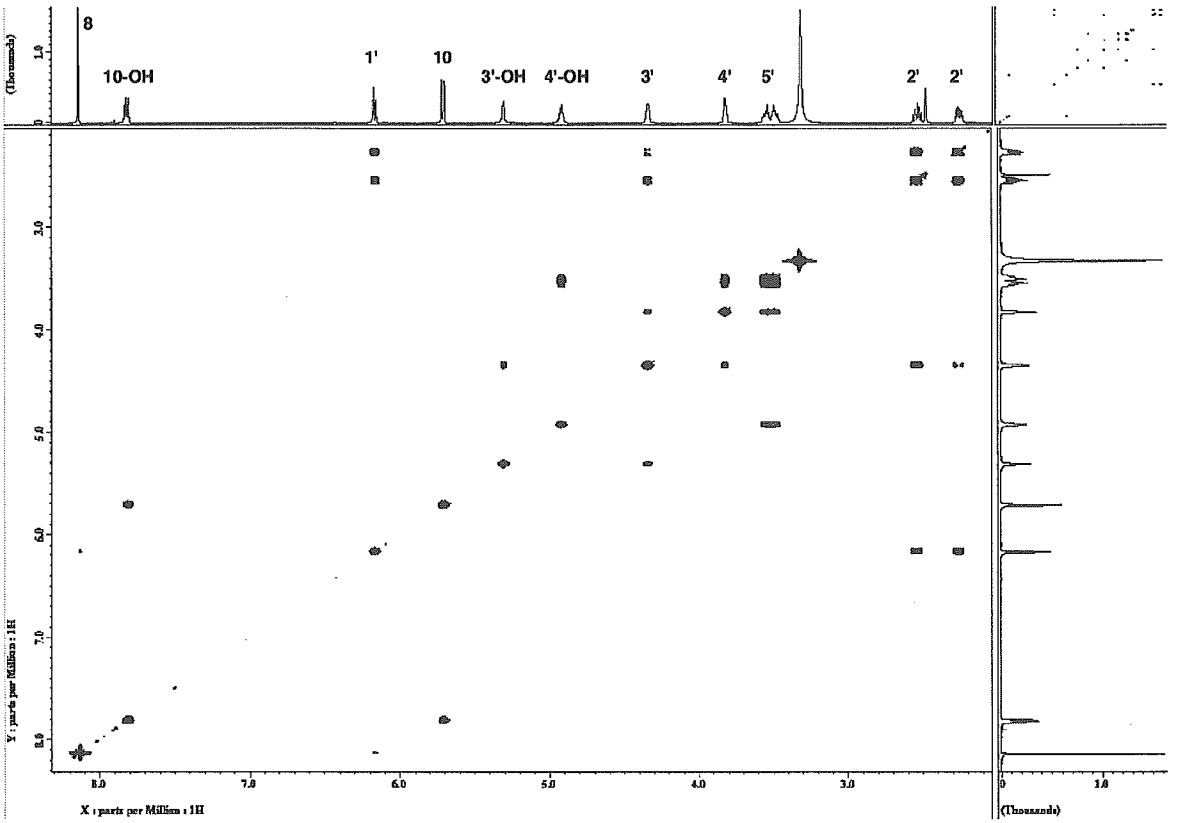


Figure 4. $^{1}H-^{1}H$ 2D COSY NMR spectrum of compound **2**.

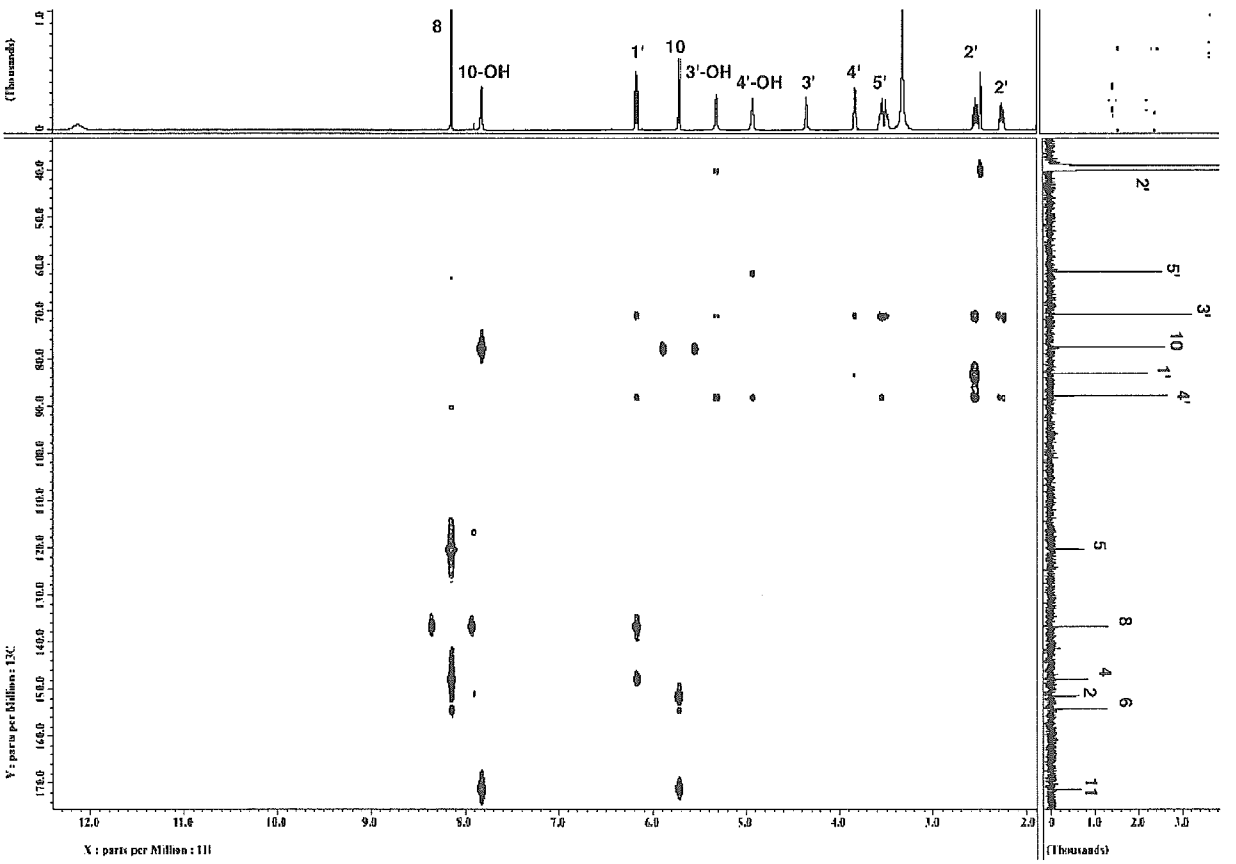


Figure 5. $^{1}H-^{13}C$ 2D HMBC NMR spectrum of compound **2**.

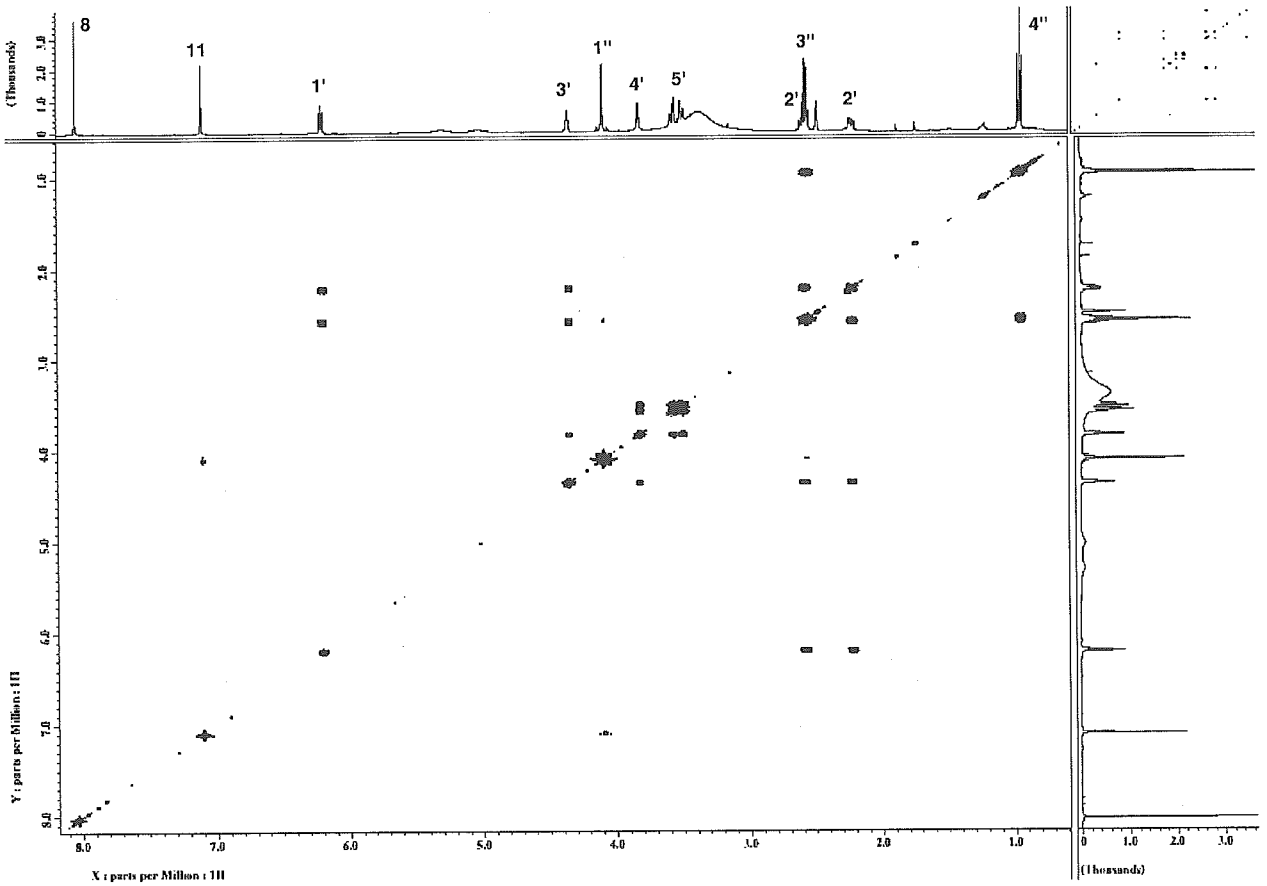


Figure 6. $^1H-^1H$ 2D COSY NMR spectrum of compound **6**.

Adduct 3 was characterized as 8-OH-dGuo, based on its two unique maxima (245 and 293 nm) in the UV spectrum (Figure 2A-3) and a comparison of its retention time in HPLC with that of authentic 8-OH-dGuo (*16*, *17*).

The mass spectra of adducts 4 and 5, $(M - H)^-$, *m/z* 352.1248 and 352.1258 (352.1257 calcd for $C_{14}H_{18}N_5O_6$), revealed an increase of 86 mass units ($C_4H_6O_2$) from dGuo. In the ^1H NMR spectrum, the presence of an ethyl group and a hemiaminal proton was observed, suggesting that they are 1,N2-cyclic ethylglyoxal-dGuo adducts. Their UV spectra (Figure 2B-4,5) are similar to those of methylglyoxal-dGuo adducts (*21*). They may be stereoisomers, because their mass, ^1H NMR, and UV spectra (Figure 2B-4,5) are identical. However, the structures of **4** and **5** were not confirmed unambiguously in the present study, because ethylglyoxal was not available.

The mass spectra of adduct 6 showed a $(M - H)^-$ ion at *m/z* 360.1308 (360.1308 calcd for $C_{16}H_{18}N_5O_5$), which corresponds to an increase of 94 mass units (C_6H_6O) from dGuo. In the ^1H NMR spectrum, the presence of an ethyl group (0.96 and 2.57 ppm), a low field methylene group (4.09 ppm), and an extra olefinic proton (7.11 ppm) was observed, in addition to the signals of the dGuo moiety. The UV spectrum of adduct 6 [λ_{max} (pH 7), 228 and 282 nm] was similar to that of 1,N2-etheno-dGuo [λ_{max} (pH 7), 228 and 283 nm], but different from that of 3,N2-ethenoguanosine [λ_{max} (pH 7), 225 and 258 nm] (*22*, *23*). The stability of the glycosyl bond of adduct 6, under conditions of 0.1% acetic acid (pH 3.2), 37 °C, and 1 h, also ruled out the possibility of the 3,N2-etheno-dGuo derivative, which is known to be rapidly hydrolyzed (half-life of 1 h) under conditions of pH 5.5, 37 °C, and 1 h (*24*). The structure of adduct 6 was proposed as a 4-oxo-2-hexenal (4-OHE)-1,N2-cyclic-dGuo adduct (structure **6**), based on these spectral data. The ^1H NMR and UV data of compound **6**, as well as the retention time in HPLC, were completely identical to those of the sole reaction product of dGuo and 4-OHE. The structure of compound **6** was confirmed by extensive NMR experiments, including COSY

Table 2. ^{13}C and ^1H NMR Assignments for Compound 6[a]

	δC (ppm)	δH (ppm)	multiplicity	*J* (Hz)
1N				
2	146.94			
3N				
4	149.79			
5	115.83			
6	153.74			
7N				
8	136.83	8.04	s	
9N				
10	117.67			
11	116.05	7.11	s	
12N				
1′	83.01	6.21	dd	7.6, 6.1
2′	39.42	2.23	ddd	13.1, 6.1, 3.1
		2.59	m	
3′	70.74	4.36	ddd	5.6, 3.1, 2.8
4′	87.64	3.83	ddd	4.7, 4.4, 2.8
5′	61.72	3.51	dd	11.7, 4.4
		3.57	dd	11.7, 4.7
3′OH				
5′OH				
1″	39.3	4.08	d	8.0
		4.11	d	8.0
2″	206.14			
3″	34.35	2.57	q	7.3
4″	7.58	0.96	t	7.3

[a] 1′–5′, numbering of sugar carbons.

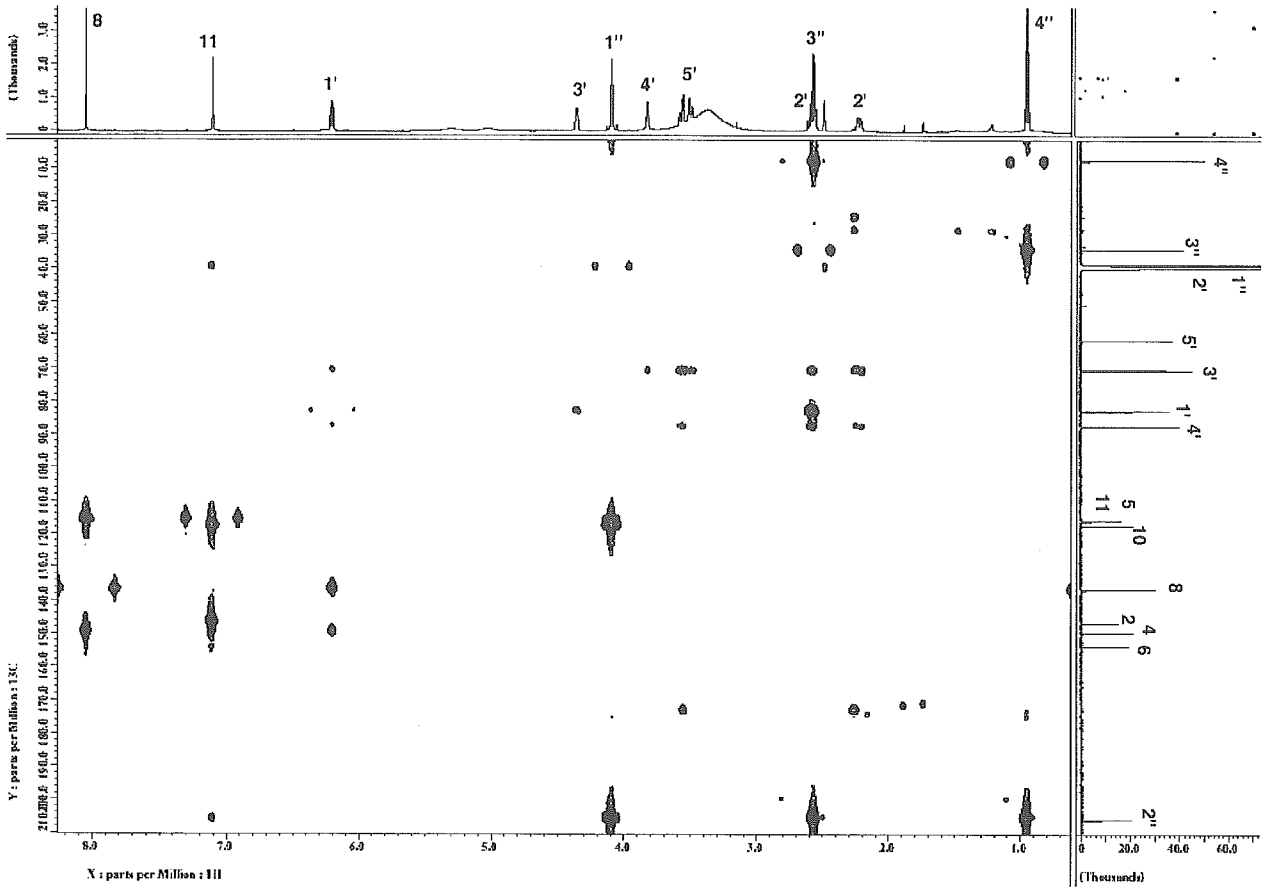
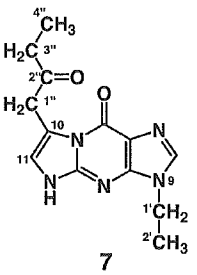


Figure 7. $^{1}H-^{13}C$ 2D HMBC NMR spectrum of compound **6**.

and HMBC, using the synthetic **6** (Table 2 and Figures 6 and 7).



7

To further confirm its structure, a highly crystalline product, 9-ethyl-10-(2-oxobutyl)-1,N2-ethenoguanine (structure **7**), was prepared from 9-ethylguanine and 4-OHE, under the same reaction conditions used for the preparation of 10-(2-oxobutyl)-1,N2-etheno-dGuo (compound **6**). Structure **7** was analyzed by X-ray crystallography (Figure 8). Because the UV spectra and the low-field ^{1}H NMR data of **6** and **7** (Tables 2 and 3) are similar, the structure of **6** is presumed to have the same ring system and substitution pattern as those of **7**.

Mutagenic Activity of 4-OHE in *Salmonella* Strains, TA100 and TA104. The mutagenicity of 4-OHE was tested at concentrations of 1.25, 2.5, 5, 7.5, and 10 μg/plate. At concentrations above 10 μg/plate, 4-OHE was quite toxic to the bacteria. 4-OHE showed mutagenic activity in the TA100 and TA104 strains, without S9 mix (Figure 9A,B, respectively). The specific mutagenic activities of 4-OHE to TA100

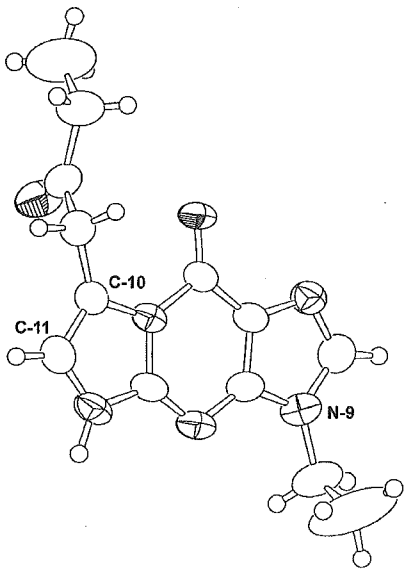


Figure 8. Crystal structure of compound **7**.

and TA104 were 78 and 67 revertants/μg, calculated from the data at the concentrations of 10 and 2.5 μg/plate, respectively. The mutagenicity of 4-OHE is also shown as the mutation frequency (revertants/ survivals) in Figure 9. 4-OHE caused a dose-dependent increase in mutation frequency in TA100 and TA104.

Table 3. ^{13}C and ^{1}H NMR Assignments for Compound 7

	δC (ppm)	δH (ppm)	multiplicity	*J* (Hz)
1N				
2	147.1			
3N				
4	150.0			
5	115.7			
6	153.8			
7N				
8	138.4	7.84	s	
9N				
10	117.5			
11	115.8	7.09	s	
12N				
1′	37.8	4.05	q	7.2
2′	15.1	1.36	t	7.2
1″	39.3	4.09	s	
2″	206.1			
3″	34.3	2.57	q	7.2
4″	7.5	0.96	t	7.2

Discussion

It is important to identify mutagens in the diet, as a prelude to cancer prevention. However, few mutagens have been identified in the human diet. It is difficult to isolate very small amounts of mutagens by HPLC from a complex mixture of chemicals, such as food, by monitoring the mutagenic activity of each fraction. For this purpose, it is more efficient to study model systems of lipid peroxidation, which may be related to high-fat and red meat diets.

In our study, the unstable mutagens present in minor amounts in the complicated mixtures of the model reactions were trapped as dGuo adducts (*16*). This method allowed us to identify mutagens, such as glyoxal, glyoxylic acid, ethylglyoxal, and 4-OHE, in the mixture. Among them, ethylglyoxal and 4-OHE have not previously been reported as mutagens.

The mutagenicity of glyoxal and methylglyoxal and their detection in various foods has been reported (*25*, *26*). Although the tumor promotion activity of glyoxal has been observed in an MNNG plus NaCl-induced stomach carcinogenesis model, glyoxal and methylglyoxal do not seem to be carcinogenic in F344 rat liver, based on a medium-term liver bioassay measuring the induction of glutathione S-transferase placental form (GST-P)-positive foci (*27*).

The mutagenicity of glyoxylic acid has also been reported as a component of an ozonated humic substance (*28*, *29*). However, its formation by lipid peroxidation has not been described. Further studies on its detection in unsaturated fatty acid-containing foods and its carcinogenicity are needed.

We detected 8-OH-dGuo in the dGuo-lipid peroxidation reaction mixture. This result is compatible with the previous observation that 8-OH-dGuo is formed from dGuo or in DNA after a reaction with autoxidized polyunsaturated fatty acids (*30*, *31*). The reason for the higher formation of 8-OH-dGuo in the MLA reaction as compared to the MLN reaction in the present study is not clear.

4-OHE may be produced via 16-hydroperoxy compound formed from MLN, followed by heme Fe-mediated decomposition, as shown in Scheme 1, by a mechanism similar to that for the formation of 4-oxo-2-nonenal (4-ONE) from 13-hydroperoxy-9,11-octadecadienoic acid (*32*). The yields of 4-OHE in the salad oil reaction (Figure 1B) and the MLN reaction (Figure 1C) from 1 mL of the corresponding oils were estimated as 12.4 and 25.8 μg, respectively, based on the peak areas of adduct 6.

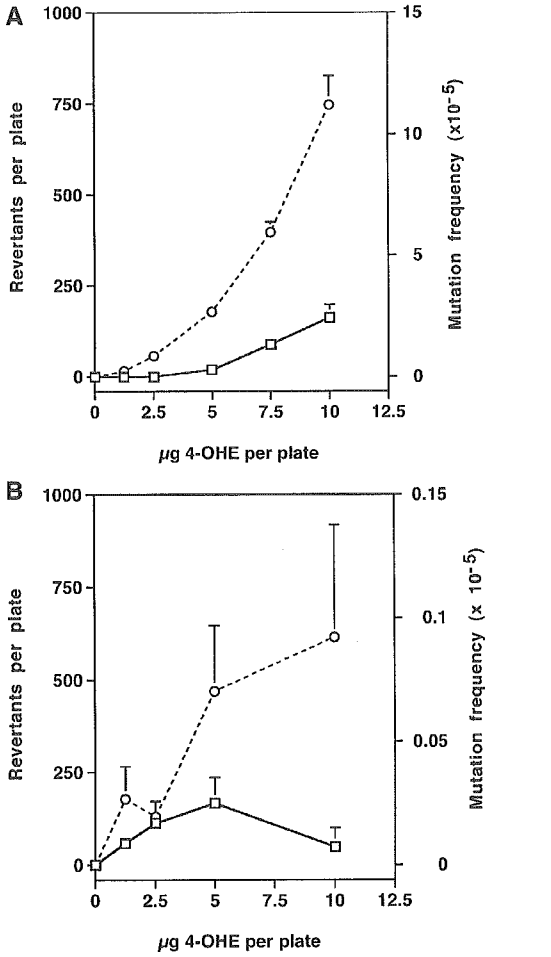
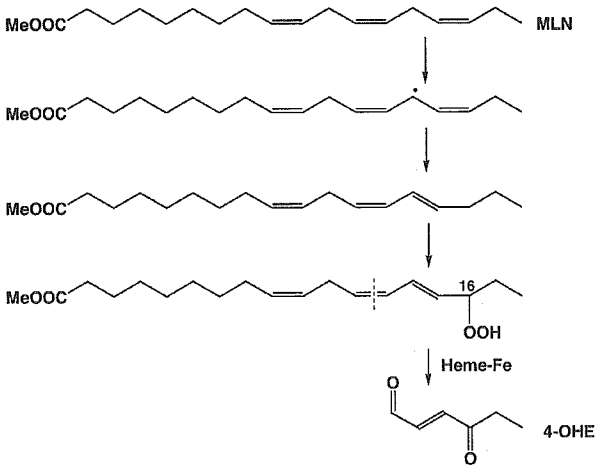


Figure 9. Mutagenicity of 4-OHE in TA100 (A) and TA104 (B). Mutagenicity (revertants/plate, —) and mutation frequency (revertants/survivals, - - -) are shown. Values represent means ± SE; $n = 4$. The number of spontaneous revertants per plate was subtracted.

Scheme 1. Mechanism for the Formation of 4-OHE from MLN



4-Hydroxy-2-nonenal and 4-ONE have been extensively studied by many researchers as the lipid peroxidation products of ω-6 fatty acids, such as linoleic acid (*32*). The structure of a 4-ONE-dGuo adduct and its detection in liver DNA from oxidative stress-induced rats have been reported (*33*, *34*). On the other hand, ω-3 fatty acids, such as α-linolenic acid,

docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), are known as important fatty acids recommended for cancer prevention, but their lipid peroxidation products, such as 4-hydroxy-2-hexenal (*35*) and 4-OHE, have not been well-studied.

The autoxidation reaction mixture (dGuo + MLN + hemin) produced more kinds and higher amounts of dGuo adducts (Figure 1C) than the (dGuo + MLA + hemin) reaction mixture (Figure 1A). This is compatible with the fact that the autoxidation rate of unsaturated fatty acids is dependent on the number of active methylene groups between the two double bonds (*36*). Therefore, more mutagens are expected to be produced by autoxidation, in the order linoleic acid < α-linolenic acid < DHA < EPA, which have two, three, four, and five active methylene groups, respectively. The different affinities of these unsaturated fatty acids for the hemin molecule may also influence the autoxidation rate. Our data are compatible with the observation that the mutagenicity of heated cooking oils correlates with the α-linolenic acid content as reported by Harris and his collaborators (*37*). We also detected a considerable amount of 4-OHE in heat-processed ω-3 fat-containing foods, such as broiled fish and perilla oil, while the amounts of 4-ONE were much lower than those of 4-OHE (Kawai et al., to be published elsewhere). In the HPLC profile of the MLA reaction mixture (Figure 1A), we could not detect an etheno-dGuo type adduct derived from 4-ONE, even after prolonged elution with an increasing acetonitrile gradient. However, we cannot rule out the possiblity that the formation of 4-ONE-dGuo adduct has not been detected, because it may be present in oil layer in the model reaction mixtures, due to its hydrophobicity.

In addition to DNA components, 4-OHE may also react with Arg, His, and Lys residues in proteins, as 4-ONE does (*38*). These modifications may exert epigenetic effects, such as modulation of transcriptional activation (*38*) or other deleterious biological phenomena. Finally, it is worth mentioning that 4-OHE has been detected as a major constituent of insect defensive secretions, which function as nonspecific irritants, toxins, or olfactory repellents of arthropods (*39*). Our results raise the concern that ω-3 fats are more toxic than ω-6 fats, in that ω-3 fats produce larger amounts of mutagens by lipid peroxidation, even if ω-3 fats themselves in the diet inhibit carcinogenesis. Further studies on the detection of glyoxylic acid, ethylglyoxal, and 4-OHE in various foods and on the carcinogenic activity of 4-OHE are now in progress in our laboratory.

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無機および有機ヒ素化合物のin vitro遺伝子突然変異誘発性と，その食物摂取からの遺伝毒性リスク

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In vitro genotoxicity of inorganic and organic arsenics and their genotoxic risk through food intake

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Summary

Arsenic compounds contained in sea foods have raised public health concerns, because their chronic exposure through dietary intake may increase cancer risk. In the present study, we investigated the in vitro genotoxicity of two inorganic arsenics (arsenite; As[III], arsenate; As[V]) and three organic arsenics (monomethylarsonic acid; MMAA, dimethylarsenic acid; DMAA, trimethylarsine oxide; TMAO) using mouse lymphoma *Tk* assay (MLA). In the standard MLA with 3 h treatment, exposure to As[III] and As[V] significantly induced *Tk*-mutants. The genotoxicity of As[III] was over 50-times greater than that of As[V]. Among organic arsenics, on the other hand, only DMAA showed weak genotoxicity with 3 h treatment at high doses. In the 24 h treatment MLA, DMAA and TMAO weakly induced *Tk*-mutants. These results indicate that inorganic arsenics rather than organic arsenics should be considered for genotoxic risk. We discussed the genotoxic risk of arsenic compounds through dietary intake.

Keywords: arsenite[III], arsenate[V], organic arsenics, mouse lymphoma *Tk* assay (MLA), genotoxic risk

緒　　言

ヒ素およびその化合物は，かつては毒薬として用いられた経緯もあり，人体に非常に有害であることはよく知られている（ATSDR, 2000）．飲み込んだ際の急性症状は，吐き気，嘔吐，下痢，激しい腹痛などが見られ，場合によってショック状態から死に至る．我が国には1955年に粉ミルクの製造過程にヒ素が混入し，それを飲んだ1万人以上の乳幼児が中毒を起こし，138名の死者を出した痛ましい事件がある（森永ヒ素ミルク事件）．慢性症状としては，剥離性の皮膚炎や色素沈着，骨髄障害，末梢性神経炎，黄疸，腎不全などがあげられる．疫学的研究からヒ素およびヒ素化合物は，発がん性も指摘されており，WHOのがん研究機構（IARC）ではヒ素をGroup I（人に対して発がん性あり）に分類している（IARC, 1987）．

ヒ素は自然にも存在しており，その管理が難しい．水

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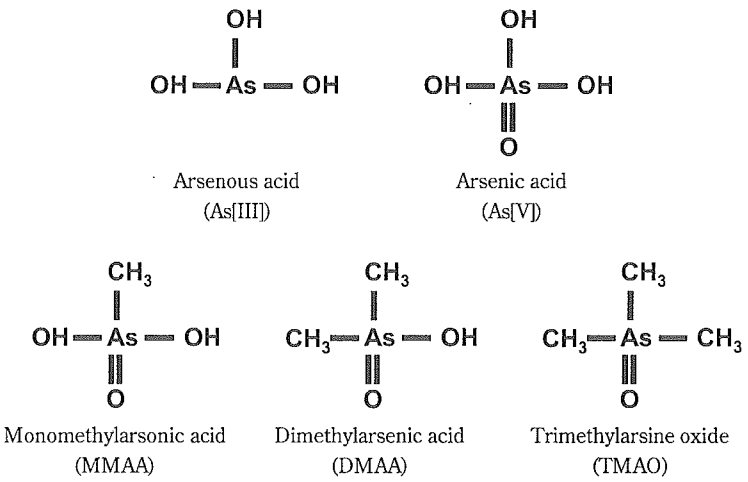

Fig. 1 Inorganic and organic arsenic compounds in marine organisms

道水中のヒ素濃度はWHOの飲料水水質ガイドラインに従い，発がんリスクをできるだけ低くするように管理されている（WHO, 2004）．また，ヒ素化合物は，生体内にごく微量が存在しており，人体にとって微量必須元素であると考えられている．ただし，生体内のヒ素の大部分は代謝により毒性の低い有機ヒ素化合物として存在している（ATSDR, 2000）．

2004年7月28日，英国食糧規格庁（Food Standard Agency）は，海草のひじき（Hijiki）に無機ヒ素（Inorganic arsenic）が大量に含まれているという調査結果に基づき，国民に対してひじきを食べないように勧告した（FSA, 2004）．海産食品にはこれまでヒ素化合物が多く含まれていることが報告されている．特に，ひじき，こんぶなどの海草類，えびなどの甲殻類，カレイなどの魚類は，ヒ素含有量が高いことが知られている（Fukui et al., 1981）．これら食品中からのヒ素化合物の摂取をできるだけ低レベルに抑えることが，水道水の管理と同様に，一般市民の発がんリスクを減らすことになるのかもしれない．

海産物中に含まれるヒ素化合物には，最も毒性が高いとされている三価の無機ヒ素（As[III]）だけでなく，五価の無機ヒ素（As[V]）や，有機ヒ素化合物などがある．そして，これらの含有量は海産物の種類によって異なることが知られている（Fukui et al., 1981）．また，これらヒ素化合物の毒性はその化学形態によって大きく異なる（ATSDR, 2000）．本研究では，2つの無機ヒ素化合物（As[III], As[V]）と，比較的海産物に多く含まれている3つの有機ヒ素化合物（モノメチルアルソン酸：monomethylarsonic acid（MMAA），ジメチルアルシン酸：dimethylarsenic acid（DMAA），トリメチルアルシンオキシド：trimethylarsine oxide（TMAO））（Fig. 1）の遺伝毒性誘発性をマウスリンフォーマ*Tk*試験（MLA）によって評価した．また，これらヒ素化合物の摂取量と，遺伝毒性の程度を，他の食品中から摂取する可能性のある化学物質のそれらと比較し，食品中からのヒ素化合物の遺伝毒性リスクを考察した．

実験材料および方法

1. 試験検体

亜ヒ酸ナトリウム（sodium arsenite（As[III]）；Cas. No. 7784-46-5, MW130）は，関東化学工業製，ヒ酸二ナトリウム（sodium arsenate（As[V]）；CAS. No. 7778-43-0, MW186）は，和光純薬工業製，特級試薬を用いた．モノメチルアルソン酸（monomethylarsonic acid（MMAA）；CAS. No. 124-58-3, MW140），ジメチルアルシン酸（dimethylarsenic acid（DMAA）；CAS. No. 75-60-5, MW138），トリメチルアルシンオキシド（trimethylarsine oxide（TMAO）；CAS. No. 4964-14-1, MW136）は，トリケミカル研究所製の純度99.9%のものを用いた．

2. 用量設定試験

細胞を試験検体で一定時間処理し，その後の48時間における細胞増殖性を，細胞数を計測して求め，陰性対照と比較した（Relative Suspension Growth; RSG）．陰性対照の10～20%になる濃度を最高用量として設定した．

3. マウスリンフォーマ*Tk*試験（MLA）

MLAはマイクロウェル法で行い，プロトコールはHonmaらの方法に従った（Honma et al., 1999a）．S9非存在下で，細胞を試験検体で3時間処理し，48時間の発現時間をおいて，*Tk*突然変異検出のために細胞をプレーティングした．細胞毒性の指標としては処理直後の相対生存率（Relative Survival; RS）と，処理後の増殖性と生存率を考慮した値（Relative Total Growth; RTG）を用いた．有機ヒ素化合物に関しては，24時間処理を実施した．24時間処理のプロトコールは，Honmaらの方法に従った（Honma et al., 1999b）．

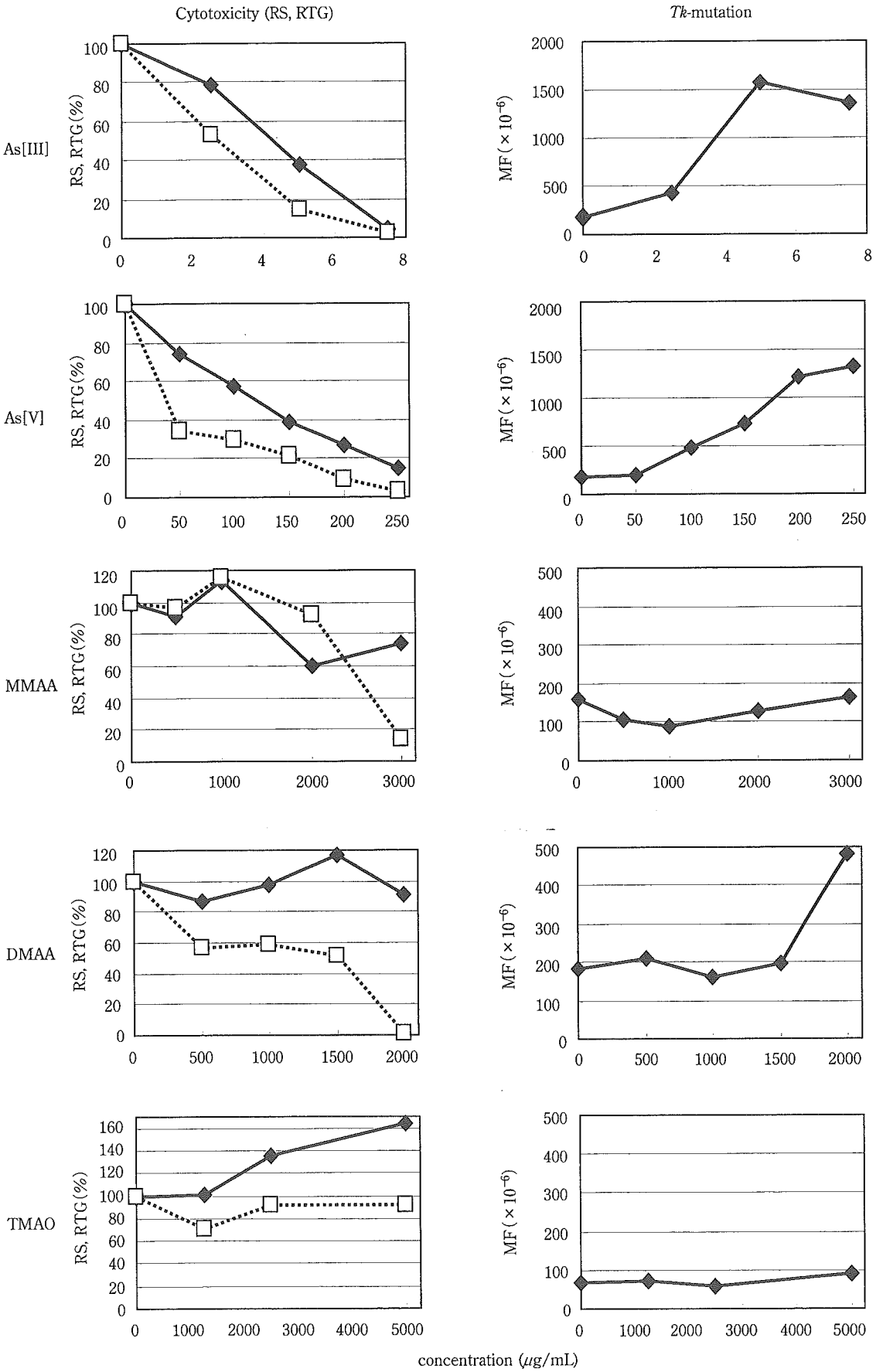

Fig. 2 Cytotoxicity (relative survival; RS, relative total growth; RTG) and *Tk*-mutation frequency (MF) in MLA treated with As[III], As[V], MMAA, DMAA, and TMAO for 3 h. In cytotoxicity, closed symbol is RS, and open symbol is RTG.

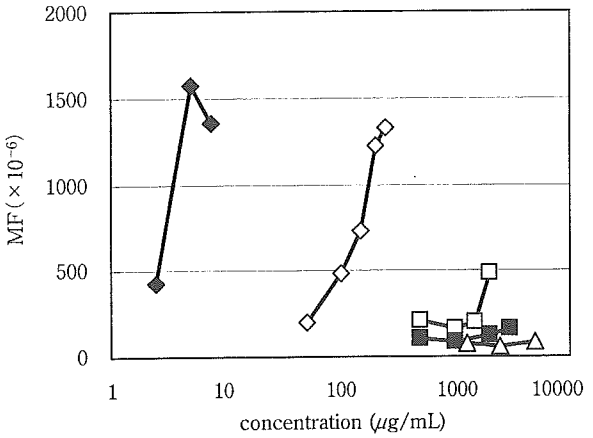

Fig. 3 Comparison of *Tk*-mutation frequencies (MF) in 3 h treatment test treated with As[III]-◆-, As[V]-◇-, MMAA-■-, DMAA-□-, and TMAO-△-.

4. 統計的解析

MLAのデータはOmoriらの統計的手法により，陽性，陰性を判定した（Omori et al., 2002）．この方法はDunnett法による陰性対照との比較検定，Simpson-Margolin法による用量依存的な傾向性検定を組み合わせた方法である．

結　　果

1. 用量設定試験

細胞を試験検体で3時間処理し，陰性対照の10～20%のRSGを示す濃度を本試験における最高用量として設定した（data not shown）．この試験より，As[III]: 10 μg/mL，As[V]: 250 μg/mL，MMAA: 3000 μg/mL，DMAA: 2500 μg/mL，TMAO: 5000 μg/mLを最高用量として設定した．TMAOは5000 μg/mLにおいてもほとんど細胞毒性を示さなかったがMLAの試験ガイドラインに従い，5000 μg/mLを最高用量とした．

2. 3時間処理試験

3時間処理によるMLAの結果をFig. 2に示す．無機ヒ素化合物のAs[III]，As[V]は用量依存的に細胞毒性（RS, RTG）を示し，それに伴い高い突然変異の誘発が認められた．統計学的にも突然変異頻度の増加は有意であった（有意水準1%）．As[III]の5 μg/mL，As[V]の250 μg/mLで陰性対照の15倍の突然変異の誘発が観察された．MLAでは，2種類の変異体（small colony mutant; SC, large colony mutant; LC）が観察されるが，これらヒ素化合物によって誘発された変異体のSC/LC比は特に陰性対照との違いは認められなかった（data not shown）．As[III]の最高用量の10 μg/mLではRS，RTGとも毒性が高すぎたため突然変異のデータは得られなかった．突然変異頻度を2倍増加させる用量（Double Mutation Frequency Dose; DMFD）はAs[III]では0.78 μg/mL，As[V]では39.5 μg/mLと計算できた．このことからAs[III]はAs[V]に比べて，用量当たり約50倍程度の遺伝毒性を持つ．

一方，有機ヒ素化合物であるMMAA，TMAOは試験濃度では全く突然変異の誘発を示さなかった．特にTMAOは細胞毒性も全く示さなかったことから，極めて毒性の低い化合物であることが予想された．DMAAは2000 μg/mLの高濃度で突然変異の増加が見られ，統計学的には有意に陽性であったが（有意水準5%），RTGが1%であり，強い細胞毒性による非特異的反応とも考えられる．2500 μg/mLでは，毒性が高すぎたため突然変異のデータは得られなかった．5つのヒ素化合物の突然変異誘発頻度の比較をFig. 3に示した．

DMAAは細胞毒性の指標であるRTGとRSに大きな差が見られた．RTGがRSに比べて低いことは強い細胞増殖阻害が起きていることを予想させる．

3. 24時間処理試験

3種類の有機ヒ素化合物は短時間処理において，無機ヒ素化合物と比較して，強い突然変異誘発性を示さなかったことから，24時間処理を実施した．3時間処理と同様に用量設定試験を実施し，最高用量を設定し，本試験を行った．本試験の結果をFig. 4に示す．全ての有機ヒ素化合物は用量依存的に細胞毒性を示した．一方，突然変異誘発性に関して，MMAAは陰性であったが，DMAA，TMAOは統計学的には陽性を示した（有意水準5%）．ただし，これら陽性反応も，RTG 5%以下の強い毒性下での反応であり，非特異的な影響であるのかもしれない．3つの有機ヒ素化合物の突然変異誘発頻度の比較をFig. 5に示した．

考　　察

1. 遺伝毒性ハザードとしてのヒ素化合物

無機ヒ素化合物の中でも毒性の高いAs[III]に関しては，多くの遺伝毒性の報告がある（Gradecka et al., 2001）．In vitro遺伝毒性においては，As[III]はエームス試験陰性を示すのに対して，培養細胞を用いた染色体異常試験，姉妹染色分体交換試験，コメット試験では陽性を示す（Basu et al., 2001; Gebel, 2001）．マウスを用いたin vivo試験では，染色体異常，小核の誘発が報告されている（Gebel, 2001）．このように，染色体レベルの強い遺伝毒性や，DNA損傷性が多数報告されているにもかかわらず，エームス試験では陰性であることから，As[III]は点突然変異を誘発するようなmutagenではなく，染色体レベルの損傷を引き起こすclastogenであるとされている（Gebel, 2001）．MLAは常染色体劣性型の遺伝子突然変異試験であり，点突然変異から，染色体レベルの変異までを検出できる広域スペクトルを持つ突然変異検出系である（Honma et al., 2001）．今回の我々の

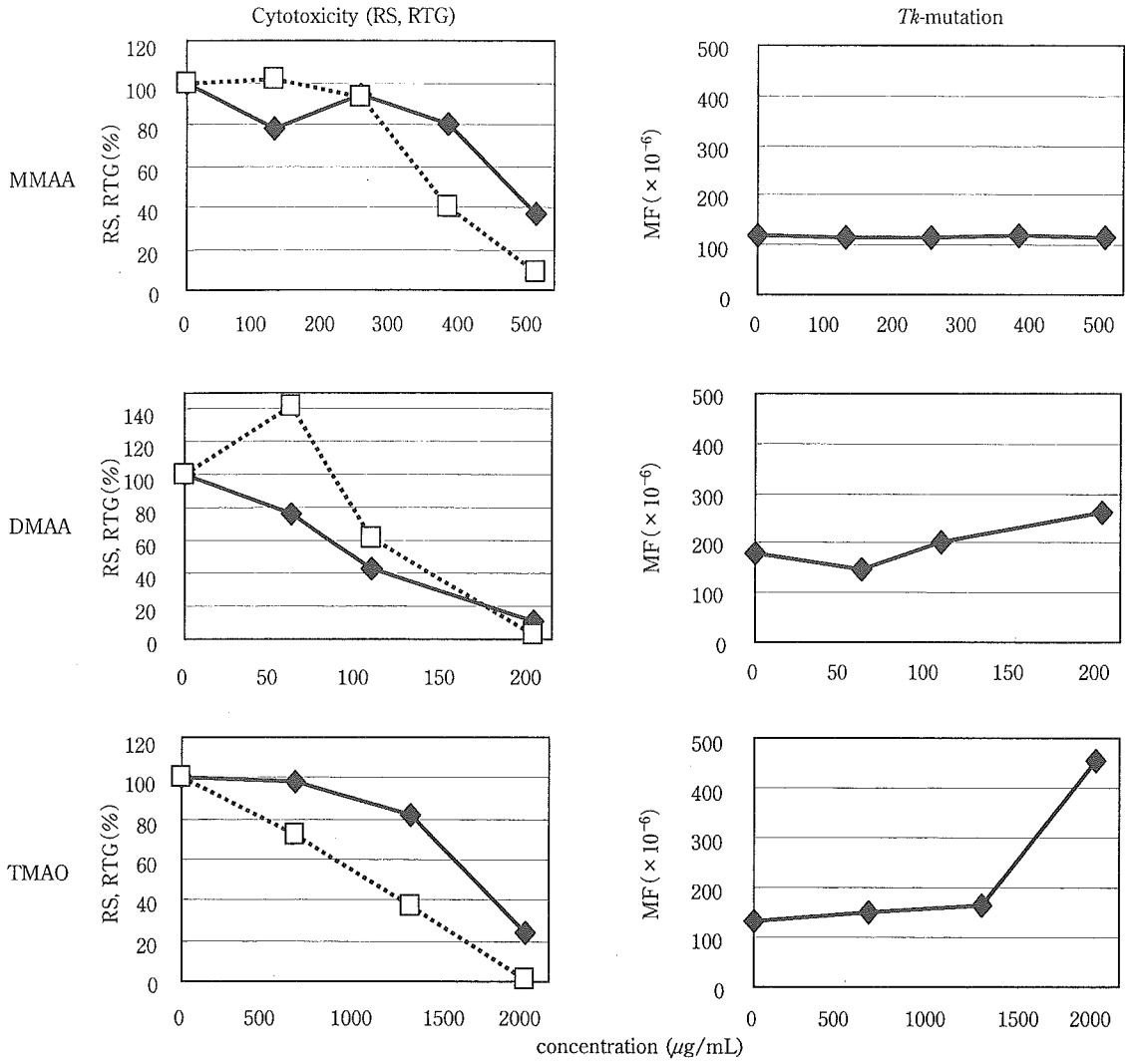

Fig. 4 Cytotoxicity (relative survival; RS, relative total growth; RTG) and *Tk*-mutation frequency (MF) in MLA treated with As MMAA, DMAA, and TMAO for 24 h. In cytotoxicity, closed symbol is RS, and open symbol is RTG.

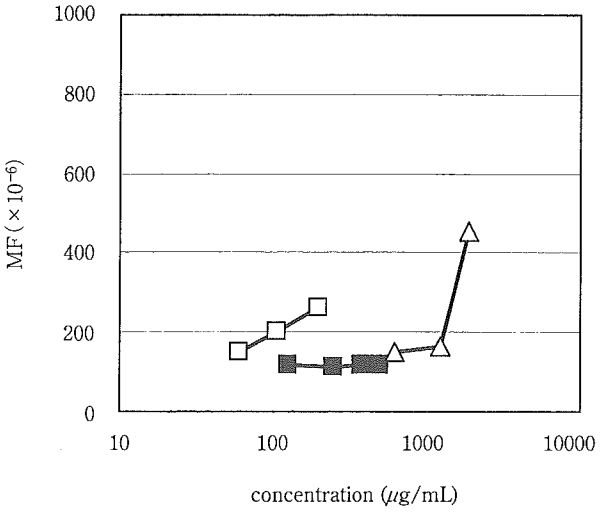

Fig. 5 Comparison of *Tk*-mutation frequencies (MF) in 24 h treatment test treated with MMAA-■-, DMAA-□-, and TMAO-△-.

研究でAs[III]がMLAで強い遺伝子突然変異誘発性を示したことは，As[III]が染色体レベルの遺伝子突然変異を引き起こすことを示唆している．MooreらもMLAでAs[III]とAs[V]の突然変異誘発性を報告している(Moore et al., 1997)．我々の結果と同様に，As[III]は，As[V]の1/10以下の試験用量で高い突然変異誘発性を示すことを報告している．

有機ヒ素化合物の遺伝毒性についての報告は多くない(Gebel, 2001; Kaise et al., 1996)．今回試験した3つの有機ヒ素化合物MMAA，DMAA，TMAOは，無機ヒ素化合物が生体内に取り込まれた後の主たる代謝産物であり，人体や，多くの食物中に存在が確認されている．解毒経路の代謝産物であり，その毒性は無機ヒ素化合物に比べて極めて低い．In vivo遺伝毒性試験において，これら有機ヒ素化合物の突然変異誘発性は陰性と報告されている（Noda et al., 2002)．今回のMLAでは，有機ヒ素化合物3種の中で3時間処理，24時間処理とも突然変異

Table 1 Human Exposure/Rodent Potency (HERP) and Human Exposure/Genotoxic Potency (HEGEP)

Compounds (Major origin)	IARC	Average daily intake (μg/day)*	HERP*	Genotoxicity			
				DMFD (μg/mL)	HEGEP	Cell, Cond.	Ref
Saccharin (Artificial sweetener)	3	95000	0.06	12000	7.9	MLA	Clive et al., 1979
Dimehtylnitrosamine (Beer)	2A	0.646	0.01	0.07	9.2	MLA, rat S9	McGregor et al., 1988
Acrylamide (Potato chips et al.)	2A	40	0.01	100	0.4	WTK-1	Unpublished data
Aflatoxin B1 (Peanut et al.)	1	0.018	0.008	0.0075	2.4	MLA, rat S9	Preisler et al., 2000
AF-2 (Preservative, -1975)	2B	4.8	0.0002	2.5	1.92	WTK-1	Unpublished data
IQ (Burnt foods)	2A	0.0064	0.00001	7.2	0.89	WTK-1	Unpublished data
Kojic acid (Miso, soy source)	2B	0.2	0.0000005	2500	0.00008	WTK-1, rat S9	Unpublished data
As[III] (Hijiki, cooked)	1	1.6	–	0.78	2.05	MLA	Present study
As[V] (Hijiki, cooked)	1	4.4	–	39.5	0.11	MLA	Present study
As[III] (Tap water and other natural sources, Max)	1	107	–	0.78	137.2	MLA	Present study

* Data from Gold et al. (2002)

誘発性が認められたのはDMAAのみであった．また，3時間処理試験での細胞毒性試験からDMAAは強い細胞増殖阻害作用を持つことが示唆された．Kashiwadaらはマウスに DMAA を腹腔内投与後，骨髄細胞において，M期での細胞周期の停止，および異数染色体細胞の増加が観察されたことを報告している（Kashiwada et al., 1998）．このような現象はコルヒチンなどの細胞分裂毒の投与で，高頻度に観察されることから，DMAAは細胞分裂毒様の作用を持つことを予想させる．MLAは細胞分裂毒による染色体の異数化を介した，染色体の脱落による突然変異も検出することができる（Honma et al., 2001）．DMAAの持つ弱い遺伝子突然変異誘発性は，このような細胞分裂毒様の作用が関係しているのかもしれない．TMAOでもRSとRTGに差が見られた．24時間処理でTMAOがわずかに突然変異誘発性を示したのは，TMAOも弱い細胞分裂毒様の作用を持つことを示しているのかもしれない．

Mooreらは，MMAA，DMAAについてもMLAを実施し，両化合物とも3000 μg/mL以上の高濃度で，弱い突然変異誘発性を認めている（Moore et al., 1997）．MMAAの結果は我々の今回の結果と異なるものであるが，試験用量や，誘発性を考慮すると，その強さは大きくはない．

これらの結果から，無機化合物であるAs[III]とAs[V]は遺伝毒性物質であるが，有機ヒ素化合物であるMMAA，DMAA，TMAOの遺伝毒性はないか，あったとしても極めて弱いものと判断できる．

2. 遺伝毒性リスクとしてのヒ素化合物

ヒ素は齧歯類を用いた発がん性試験では陰性を示すが，疫学的研究から発がん性が明らかであるため，IARCではGroup I（人に対して発がん性あり）に分類されている（IARC, 1987）．ヒ素は我々の環境中に普遍的に存在する．そのため水道水中のヒ素濃度は，発がんリスクをできるだけ低減化させるため管理されている．現在，水道水中のヒ素濃度の基準は0.01 mg/Lである（WHO, 2004）．この濃度の発がん率は10万人中60人であり，他の環境発がん物質の基準の10倍以上も高い．これは，ヒ素が自然由来であり，その管理が困難であるためのやむを得ない措置と言える．

2004年7月28日に英国食糧規格庁（Food Standard Agency）は，海草のひじき（Hijiki）に無機ヒ素化合物が大量に含まれている理由で，国民に対してひじきを食べないように勧告した（FSA, 2004）．ひじきには乾燥重量当たり総ヒ素量として36～80 μg/gのヒ素化合物が含まれており（鴨志田ら，2005），水抽出物からの成分分析により，その83%が無機ヒ素化合物と報告されている（As[III]；22%，As[V]；61%）（Fukui et al., 1981）．他の多くの海産物にもヒ素が含まれるが，無機ヒ素の含有量はひじきが圧倒的に高い．しかし，ひじきは通常の調理過程（水戻し，加熱）で，約90%のヒ素が流出することが知られている（鴨志田ら，2005）．日本人のひ

じきの1日平均消費量は0.9 gであり（MHLW, 2004），仮に80 μg/gのヒ素を含むひじきを調理し，食したとすると，1日当たり約6 μg の無機ヒ素を摂取する計算になる（As[III]；1.6 μg，As[V]；4.4 μg）．WHOが定めた無機ヒ素のPTWI（暫定的耐用週間摂取量）は15 μg/kg/weekであるが，これは体重50 kgの人で，1日当たり107 μgのヒ素に相当する．このことから，日本人平均の18倍以上のひじきを食さない限り，PTWIを超えることはない．従って，バランスのよい食生活を心がければ，ひじきによる健康上のリスクは高まることがないと考えられる．

日常生活において摂取が避けられない発がん物質のリスク評価として，GoldらはHuman Exposure/Rodent Potency Index; HERPの利用を提唱している（Gold et al., 1990）．これは，人がその発がん物質暴露する体重当たりの平均暴露量を，その物質が齧歯類を用いた発がん性試験において動物の半数にがんを引き起こす量（TD50）で割ったものである．従って，前者が大きければ大きいほど，後者が小さければ小さいほど，HERPの値は大きくなり，そのリスクも高いと判断される．いくつかの例をTable 1に示した．HERP値の絶対量は1を基準として，それより高いものは，人での暴露が，動物実験でのTD50を引き起こす暴露量を超えることを示唆しており，また，相対的な数値は日常生活における発がんリスクのランキングを示す．しかしながら，HERP値はTD50を基礎としているため，齧歯類発がん性試験において陰性を示す物質については適用できない．実際に海産物から摂取されるヒ素のHERP値は計算されていない．この場合，齧歯類発がん性試験に替わる定量的データとして，遺伝毒性試験データを使い，HERPと同様にHuman Exposure/Genotoxic Potency; HEGEPを算出することを提案したい．ここでは，人がその発がん物質暴露する平均1日暴露量（μg/day）を，MLA，もしくはそれに準ずる遺伝子突然変異試験において，その発がん物質暴露が2倍の突然変異誘発率を引き起こす濃度（Double Mutation Frequency Dose; DMFD（μg/mL））で割ったものである．HEGEP値はHERPと異なり，その絶対値は生物学的な意味を持たない．また，in vitro試験データと，人の平均1日暴露量（μg/day）を組み合わせることは，個々の物質の遺伝毒性の発現メカニズムを無視した方法である．しかしながら，多くの遺伝毒性物質のHEGEPを，HERPと同様にランキングすることにより，他の遺伝毒性物質との相対的リスクをある程度理解することには有効であると考えられる．HEGEPの計算には，定量的な遺伝毒性試験パラメータであれば何でも利用でき，たとえば，染色体異常試験のD20値を基礎としたHEGEPも算出可能である．HEGEPの計算例もTable 1に示した．ここでは，MLA，もしくはそれと同程度の検出感度を持つWTK-1細胞による*Tk*突然変異試験でのDMFDを定量パラメータとしてHEGEPを計算した．

調理されたひじきから摂取される無機ヒ素量は先の計算からAs[III]が1.6 μg，As[V]が4.4 μgと算出できる．毒性が高いAs[III]のHEGEPは2.05であり，この遺伝毒性リスクはピーナッツ等からのaflatoxin B1（2.4）や，焦げた食品からのIQ（0.89）とほぼ同程度であり，日常生活において特に際立って遺伝毒性のリスクを増加させるとは考えられない．また，水道水等から日常生活において，別に70倍近くものAs[III]を摂取している可能性があり，それを考慮しても，ひじきから摂取しうるAs[III]の遺伝毒性リスクは，日常生活を極端に脅かすものではないと考えられる．

このように，日常生活中に受ける可能性のある遺伝毒性リスクをHEGEPとして算出し，他の物質と相対的に評価することは，そのリスクを理解しやすい．HERPとHEGEPの値を考慮し，発がん性，遺伝毒性リスクを総合的に評価することは極めて現実的な手法と考えられる．

結　論

ヒ素化合物の中で，無機化合物であるAs[III]，As[V]は，明らかに遺伝毒性物質である．ひじき中にはこれら無機ヒ素化合物が比較的多く含まれているが，その平均摂取量，および水道水等の他の摂取要因のレベルを考慮すると，ひじき食を介して摂取するヒ素化合物の遺伝毒性リスクは大きくないものと考えられる．

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3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone [MX] shows initiating and promoting activities in a two-stage BALB/c 3T3 cell transformation assay

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A transformation assay using BALB/c 3T3 cells was conducted on 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) to assess initiation and promotion activities of MX carcinogenesis. Statistically significant positive responses were obtained compared with the corresponding solvent controls in both the initiation assay post-treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and the promotion assay pretreated with 3-methylcholanthrene (MCA). Both TPA and MX inhibited metabolic cooperation in an assay using co-culture of V79 6-thioguanine (6-TG) sensitive and insensitive cells. However, cells isolated from transformed foci in the initiation assay did not induce any nodules after inoculation to BALB/c mice, the strain of mouse from which the transformation assay cells were derived. Although the study was carried out for 2–3 weeks, this might have been too short to develop nodules under the conditions of this experiment. This *in vitro* cell transformation study with MX adds supportive information to studies showing MX carcinogenicity and tumour promoter activity, and adds mechanistic understanding of the action of MX.

Introduction

3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone(MX), an organic chlorinated contaminant commonly found in tap water as a disinfection by-product (1), induces malignant tumours in the rat thyroid and mammary glands (2) and shows promoting activity in the rat stomach (3). Concerning mutagenicity, MX shows positive results in several *in vitro* assays, including the bacterial reverse mutation assay (Ames test) and the cultured cell mutation assay regardless of the presence of an exogenous metabolic activation system. Therefore, MX is classified as a direct acting mutagen (4–9). However, conflicting results have been reported for MX activity in *in vivo* genotoxicity assays (4,5,10–12). We have also performed several *in vivo* assays, namely, detection of 8-hydroxy-deoxyguanosine formation, gene mutation assay using transgenic mice (Muta™Mouse), and the comet assay, and almost all of the assays gave negative responses (M. Nakajima, S. Masumori, M. Kikuchi, S. Inagaki, J. Tanaka, Y. Furuya, M. Hayashi and N. Kinae, manuscript in preparation). Thus, the genotoxicity findings have not provided a conclusive mechanism for MX carcinogenicity.

Among the several types of cell transformation assays, we selected the focus assay using the BALB/c 3T3 established cell line for the present study because of its good reproducibility (13) and because the assay has been widely used in the elucidation of carcinogenicity mechanisms. We used A31-1-1 clone, isolated by Kakunaga and Crow (14) and thought to be sensitive to many chemicals, and employed a protocol with modified culture medium and shortened exposure period (15). The modified method can assess the potential initiation and promotion activities of test chemicals.

Many tumour promoters inhibit gap junctional intercellular communication (GJIC). For example, phorbol esters such as the strong promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) inhibit GJIC in metabolic cooperation assays (16–18). As such, the metabolic cooperation assay has been widely used as a tool for detection of promoters and for providing information about the mechanism of carcinogenicity. Therefore, we applied the assay using V79 cells to assess the promoting potential of MX.

Materials and methods

Test compounds and positive control substance

MX (CAS No. 77439-76-0, purity: 99.2%) was synthesized at the Laboratory of Food Hygiene, University of Shizuoka according to a modified method (19). It was dissolved in physiological saline, Japanese Pharmacopoeia (Otsuka Pharmaceutical Factory, Inc.). 3-Methylcholanthrene (CAS No. 56-49-5; MCA; Wako Pure Chemical Industries Ltd, Osaka, Japan), the initiator, was dissolved in dimethylsulfoxide (DMSO; purity: 99.7% or higher: Merck KGaA, Darmstadt, Germany) and used at a concentration of 0.2 μg/ml. TPA (CAS No. 16561-29-8; Wako Pure Chemical Industries Ltd), the promoter, was dissolved in DMSO and used at 0.1 μg/ml.

Preparation of test cells and culture medium

BALB/c 3T3 A31-1-1 cells were supplied by Showa Denko K.K. Cells were cultured in a CO_2 incubator (FORMA and SANYO Electric Medica Systems Co. Ltd) under 5% CO_2 atmosphere at 37°C. Eagles-MEM liquid medium (Asahi Techno Glass Corporation, Funabashi, Chiba) supplemented with 10% fetal bovine serum (FBS; Moregate BioTech, Bulimba, Australia) and 60 mg/ml Kanamycin sulfate (Invitrogen Corp., NY) (MEM) was used throughout the experiment unless otherwise indicated. Low serum concentration medium (DMEM:F12) used was DMEM:HAM's F-12 liquid medium (Asahi Techno Glass Corporation) supplemented with 5.2 ml Daigo's ITES (insulin, transferrin, ethanolamine, sodium selenite; Wako Pure Chemical Industries Ltd) per 500 ml and 2% FBS. The 500 ml extracting medium for absorption measurement was composed of 4.48 g sodium citrate dihydrate, 97.5 ml of 0.1 mol/l HCl, and 250 ml ethanol made to 500 ml with distilled water. For the metabolic cooperation assay using V79 cells, the medium used was Eagles-MEM liquid medium supplemented with 3% fetal bovine serum, 0.1% Eagle's non-essential amino acids (Invitrogen Corp.), 0.1% pyruvic acid and 0.1% glutamic acid.

Dose range-finding cytotoxicity test

For cytotoxicity testing with the initiation assay protocol, 1×10^3 cells were seeded into 24-well plates and treated 24 h later with MX at 1.42, 1.90, 2.53,

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3.38, 4.50, 6.00 and 8.00 μg/ml. The culture medium was removed 72 h later, and the cells were fixed with 10% neutral buffered formalin and stained with 0.1% crystal violet for 30 min. For cytotoxicity testing with the promotion assay protocol, cells were seeded as above and MEM was replaced with DME/F12 medium 48 h after seeding. The cells were then treated with 1.68, 2.10, 2.62, 3.28, 4.10, 5.12 and 6.40 μg/ml of MX, and fixed and stained 96 h after cell seeding in the same manner as for the initiation assay cytotoxicity test. Extractant (1.5 ml) was placed in each well for 10 min and then absorption was measured with a spectrophotometer set at 580 nm. Cell survival at each dose was calculated relative to the negative vehicle control group. The MX concentration that inhibited cell growth by 50% (IC_{50} value) was calculated using the Probit method and approximately twice the IC_{50} was selected as the highest concentration for the cell transformation assays.

Cell transformation assays

In the initiation assay, 1.2×10^4 cells were seeded into 60 mm diameter culture dishes, 12 dishes per concentration, and control groups. After 24 h incubation, DMSO as negative control, MX as the initiator (1.64, 2.05, 2.56, 3.20 and 4.00 μg/ml) or MCA as a positive control (0.2 μg/ml) was added. Seventy-two hours after treatment, MEM was replaced with fresh DME/F12. On the 7th day after the beginning of treatment, TPA (0.1 μg/ml) or DMSO was added to cultures as the first promoter treatment. For the second and third promoter treatments, TPA or DMSO was added on the 11th and 14th day, respectively.

In the promotion assay, MCA (0.2 μg/ml, as the initiator) or DMSO was added 24 h after seeding 1.2×10^4 cells per dish. On the 4th day, MEM was replaced with fresh DME/F12. On the 7th day, saline, MX (0.156, 0.313, 0.625, 1.25 and 2.50 μg/ml) or TPA (positive control; 0.1 μg/ml) was added to the culture. MX and TPA were also added on the 11th and 14th day.

The cells were fixed with methanol and stained with 2.5% Giemsa solution on the 25th day for both assays. The foci that met the following criteria were counted as transformant: (i) 2 mm or more in diameter, (ii) criss-cross growth pattern, (iii) layering of cells and (iv) deep basophilic staining.

Tumourigenicity of transformed cells

Six-week-old male BALB/c CR mice were purchased from Japan Slc, Inc. (Shizuoka, Japan), and were quarantined and acclimated to the testing facility for 1 week. They were given pelleted diet (MF: Oriental Yeast Co., Ltd) and tap water *ad libitum* through the acclimation and assay periods.

At the end of the transformation assay with MX (4.0 μg/ml as initiator and 2.5 μg/ml as promoter; experimental data not shown), cultures were washed once with Dulbecco's PBS. Cells were isolated from transformed foci by trypsinization and mass cultured. An aliquot of 0.2 ml of cell suspension (1×10^6 cells for Experiment 1 and 1.5×10^6 cells for Experiment 2) was injected subcutaneously into the cervical region of the BALB/c CR mice. In both experiments, the cells isolated from the transformed foci in the negative control groups were inoculated into three animals and cells isolated from MX-induced transformed foci were inoculated into four animals. All animals were examined 2 weeks (Experiment 1) or 3 weeks (Experiment 2) after inoculation.

Metabolic cooperation assay

6-Thioguanine (6-TG) sensitive V79 cells (6-TG^s; 4×10^5 cells) and 6-TG resistant cells (6-TG^r; 200 cells) were co-cultured to evaluate the inhibition of metabolic cooperation (5 dishes per concentration). For calculating cytotoxicity 200 V79 [6-TG^s or 6-TG^r] cells alone were plated (3 dishes per concentration). Cells were treated with either MX or TPA 4 h after seeding. The 6-TG (10 μg/ml) was added 15 min after MX or TPA treatment and cells cultured for an additional 3 days before the medium was replaced with fresh medium containing only 6-TG; the cells were cultured another 4 days. The cells were fixed in ethanol and stained with 0.1% crystal violet for 10 min. Colonies with 50 or more cells were counted. These colonies developed from cells that were either not in GJIC contact with 6-TG^s cells or were in contact but then 'rescued' by GJIC inhibition from test chemical action. The assay is based on toxicity of 6-TG to 6-TG^sV79 cells ($HGPRT^+$), non-toxicity of 6-TG to 6-TG^r mutant V79 cells ($HGPRT^-$), with toxicity to these latter cells if in GJIC contact with $HGPRT^+$ cells, which transfer the HGPRT-catalysed toxic 6-TG metabolite via gap junctions to the $HGPRT^-$ cells. Inhibition of GJIC rescues the contacting mutant cells to allow their clonal expansion (20).

Statistical analysis

The percentage of dishes with foci and the mean number of foci per dish were analysed using Fisher's exact test and the Wilcoxon's rank sum test, respectively.

In the metabolic cooperation assay, the number of 6-TG^r colonies was analysed for difference from the negative control group using Dunnett's test.

Results

Initiation assay

In the cytotoxicity assay for dose-range finding, a concentration-dependent decrease in cell survival was observed with MX treatment (Figure 1). An IC_{50} value using Probit's method was calculated to be 1.92 μg/ml. In the negative control group (saline initiation–TPA promotion) the mean number of transformed foci per dish was 0.50 and the percentage of dishes with foci was 41.7% (5 of 12 dishes, Table I). When MX was used as an initiator at 1.64, 2.05, 2.56, 3.20 and 4.00 μg/ml, and with DMSO post-treatment, no significant increase in transformation was observed. In the five groups treated with 1.64–4.00 μg/ml MX (as initiator) and TPA (0.1 μg/ml, as promoter) the numbers of foci per dish were

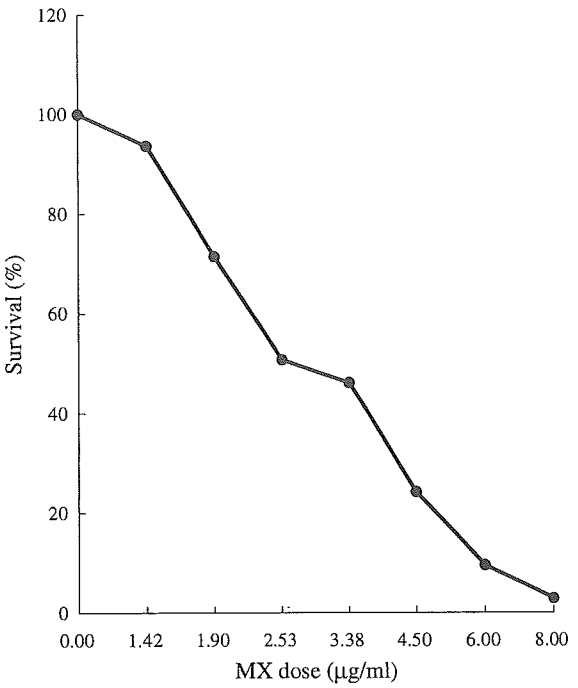

Fig. 1. MX dose–response for cell survival (Initiation protocol).

Table I. Initiating activity of MX in the two-stage transformation assay

Initiator	Conc. (μg/ml)	Survival (%)	Promoter	Conc. (μg/ml)	Mean foci/dish	Number of dishes with foci
Saline		100.0	DMSO		0.09	1/11 (9.1%)
MX	1.64	73.8	DMSO		0.08	1/12 (8.3%)
	2.05	59.2			0.25	2/12 (16.7%)
	2.56	33.7			0.00	0/12 (0.0%)
	3.20	20.5			0.33	3/12 (25.0%)
	4.00	18.8			0.25	3/12 (25.0%)
MCA	0.2	57.6	DMSO		0.58*	7/12* (58.3%)
Saline		–	TPA	0.1	0.50	5/12 (41.7%)
MX	1.64	–	TPA	0.1	0.58	6/12 (50.0%)
	2.05	–			1.25	8/12 (66.7%)
	2.56	–			1.92*	10/12 (83.3%)
	3.20	–			1.82*	9/11 (81.8%)
	4.00	–			3.64**	11/11** (100%)
MCA	0.2	–	TPA	0.1	7.58**	12/12** (100%)

*$P \leq 0.05$, **$P \leq 0.01$, significant difference from control (Wilcoxon's rank sum test for mean number of foci and Fisher's exact test for the percentage of dishes with foci).
Conc., concentration.

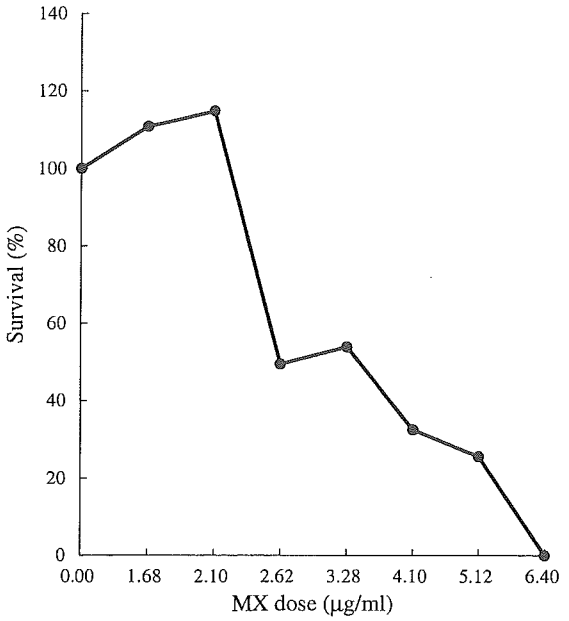

Fig. 2. MX dose–response for cell survival (Promotion protocol).

0.58, 1.25, 1.92, 1.82 and 3.64, respectively. A statistically significant increase ($P < 0.05$) was observed at MX ≥2.56 μg/ml compared with the negative control group. The number of dishes with foci also increased in a concentration-dependent manner and foci were observed in all dishes at the highest concentration (4.00 μg/ml) group. A large number of foci were induced in the positive control and MCA initiation-TPA promotion group; the mean number of foci per dish was 7.58, and all dishes contained foci.

Promotion assay

In the cytotoxicity assay, a concentration-dependent decrease in cell survival with MX treatment was observed (Figure 2). The IC_{50} value was 3.37 μg/ml. The experimental doses for promotion assay included at least 2 doses expecting to have ≥90% cell survival rate. In the negative control group (MCA initiation–saline promotion), the mean number of foci per dish was 0.25 and the percentage of dishes with foci was 25% (3 out of 12 dishes, Table II). After initiation treatment with DMSO, cells were treated with MX at 0.156, 0.313, 0.625, 1.25 and 2.50 μg/ml as the promoter. The numbers of foci were 0.00, 0.08, 0.00, 1.10 and 0.90 per dish, respectively, with only the highest dose eliciting a significant increase in dishes with foci (Table II). In the groups treated with MCA (0.2 μg/ml) and MX at the above five concentrations, the numbers of foci were 0.25, 0.92, 1.33, 3.40 and 6.18 per dish, respectively. A statistically significant increase ($P < 0.05$) was observed at concentrations ≥0.625 μg/ml compared with the negative control group. The number of dishes with foci also increased concentration-dependently, and foci were observed in all dishes of the 1.25 and 2.50 μg/ml groups. The positive control group (MCA initiation–TPA promotion) confirmed the effectiveness of MCA/TPA in this cell transformation assay.

Tumourigenicity assay

Gross examination of the mice necropsied in tumourigenicity experiments 1 and 2 did not reveal any visible nodules or tissue masses in any organs of any animals.

Table II. Promoting activity of MX in the two-stage transformation assay

Initiator	Conc. (μg/ml)	Promoter	Conc. (μg/ml)	Survival (%)	Mean foci/dish	Number of dishes with foci
DMSO		Saline		100.0	0.08	1/12 (8.3%)
DMSO		MX	0.156	98.5	0.00	0/12 (0.0%)
			0.313	103.2	0.08	1/12 (8.3%)
			0.625	93.3	0.00	0/12 (0.0%)
			1.25	90.6	1.10	4/10 (40.0%)
			2.50	90.2	0.90**	7/10** (70.0%)
MCA	0.2	Saline		–	0.25	3/12 (25.0%)
DMSO		TPA	0.1	160.8	0.25	3/12 (25.0%)
MCA	0.2	MX	0.156	–	0.25	3/12 (25.0%)
			0.313	–	0.92	5/12 (41.7%)
			0.625	–	1.33*	10/12** (83.3%)
			1.25	–	3.40**	10/10** (100%)
			2.50	–	6.18**	11/11** (100%)
MCA	0.2	TPA	0.1	–	2.73**	10/11** (90.9%)

*$P \leq 0.05$, **$P \leq 0.01$, significant difference from control (Wilcoxon rank sum test for mean number of foci and Fisher's exact test for the percentage of dishes with foci).
Conc., concentration.

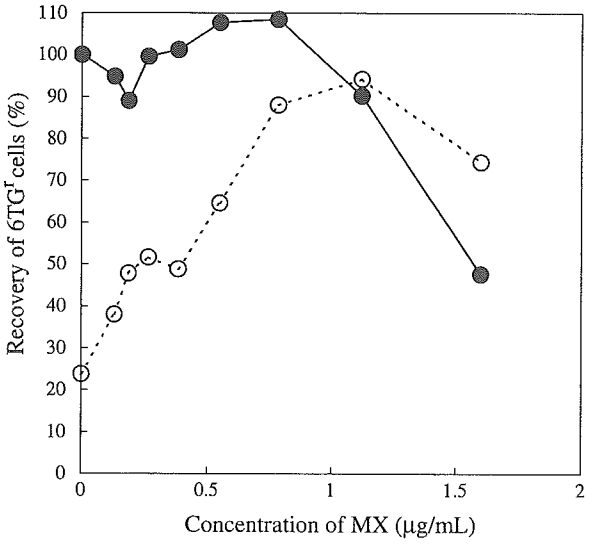

Fig. 3. Inhibition of intercellular metabolic cooperation in V79 cells by MX. For calculation of cell survival, 200 6-TG^r cells were cultured (filled circle). Recovery of 6-TG^r cells (open circle) under conditions of metabolic cooperation (4×10^5 6-TG^s cells and 200 6-TG^r cells).

Inhibition of metabolic cooperation assay

The mean number of 6-TG^r colonies increased dose-dependently in the MX-treated groups (Figure 3), indicating an inhibition of GJIC by MX. This occurred at non-cytotoxic concentrations. The mean number of 6-TG^r colonies at 1.12 μg/ml of MX was 188.4 (=94.2%) compared with 47.6 (=23.8%) in the negative control. In the positive control (TPA-treated) group the mean number of 6-TG^r colonies was 159.4 (=79.7%).

Discussion

From a public health viewpoint it is important to understand the toxicology of MX. This is particularly evident since long-term animal studies have shown carcinogenic and tumour promoting activity of MX (2,3). In the present study, we conducted transformation assays on MX using BALB/c 3T3 cells to give