Watanabe found the floc density function that can be express as equation (2) (Tambo and Watanabe, 1979, Tambo 1991).

$$\rho_e = \frac{a}{(d/1)^K} \tag{2}$$

where a and K: coefficients of the floc density function (g/cm^3 and non-dimensional, respectively) and 1: a unit to adjust dimension (cm).

It is confirmed that this function can be applied to PSI. As for the aluminium coagulants, floc density was not affected by pH but greatly affected by AlT ratio (Tambo and Watanabe, 1979). But in the case of PSI, either FeT ratio, pH, or rapid mixing speed has little effect on floc density (plots on Figure 2 include the results under various pH and rapid mixing speed conditions).

Figure 3 shows the floc density functions derived by ferric coagulants (PSI and $FeCl_3$) and alum (AlT ratio 1:20). At the same floc size, floc density of ferric coagulants is much higher than that of alum. Additionally, floc density functions of all ferric coagulants in this experiment, *i.e.* PSI-1, 3, 5 and $FeCl_3$, can be expressed by almost the same equation.

Floc growth pattern over time

Floc size distributions stirring at 300 rpm for designated time, are shown in Figure 4 (PSI-1) and Figure 5 (alum). Lines in these figures are based on log-normalized probability plots.

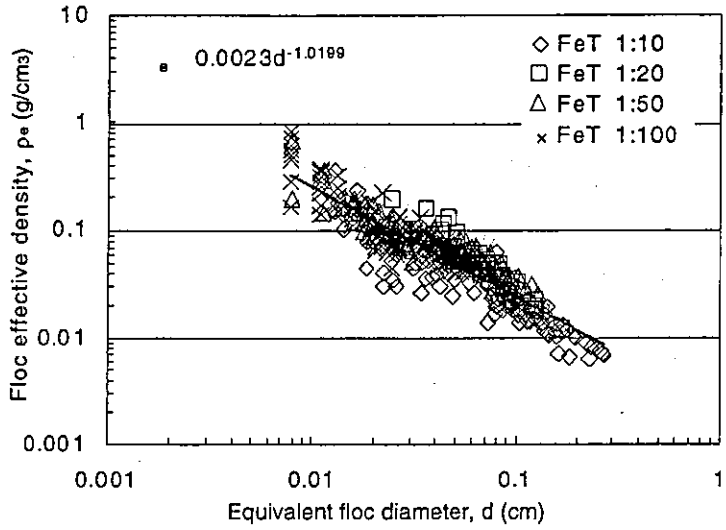

Figure 2 Plot of floc density function (PSI-1)

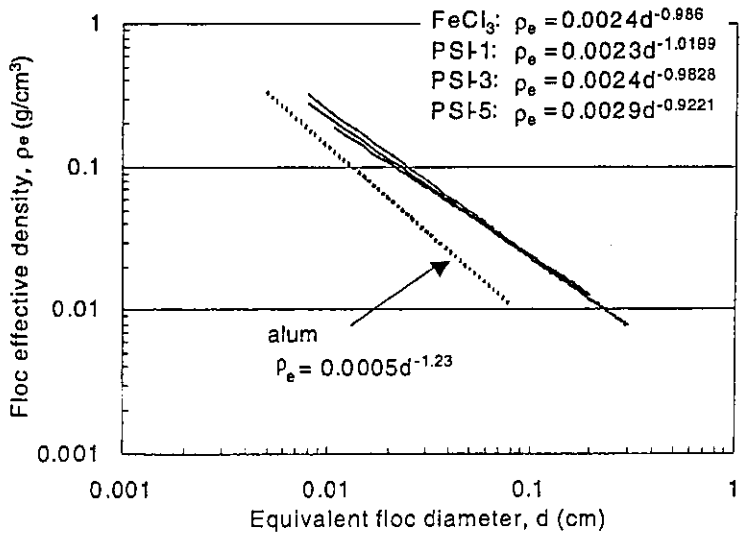

Figure 3 Comparison of floc density function on several coagulants

Thin lines are the size distributions of kaolinite clay particles in test water, and thick lines are those of formed flocs after designated stirring time. As shown in Figure 4, PSI-1 is strong enough to produce well grown flocs at the stirring speed of 300 rpm and the best-grown flocs are formed after 7 minutes of stirring. Flocs after 60 minute stirring are smaller than those after 7 minute stirring. This indicates that breakdown of flocs occurs at excessive stirring time. In the case of alum, this stirring speed is too fast to make well grown flocs and many micro flocs remain although a part of them become large flocs (Figure 5).

To confirm the removal ability, residual turbidity after sedimentation was measured. Results are shown in Figure 6. Stirring at 300 rpm for 7 minutes, residual turbidity becomes 0.35 mg/L as kaolinite clay after 15 minute sedimentation, which shows better removal than rapid mixing at 120 rpm for 5 minutes followed by slow mixing at 40 rpm for 25 minutes. Therefore, it can be said that PSI produces well grown flocs at the stirring speed of 300 rpm and does not require slow mixing process that is essential for aluminium coagulants.

Strength of floc

Relationship between the effective energy dissipation rate, ε_0 (W/m^3) and the maximum floc diameter, d_{max} (cm) is shown in Figure 7. To plot this figure, relationship between

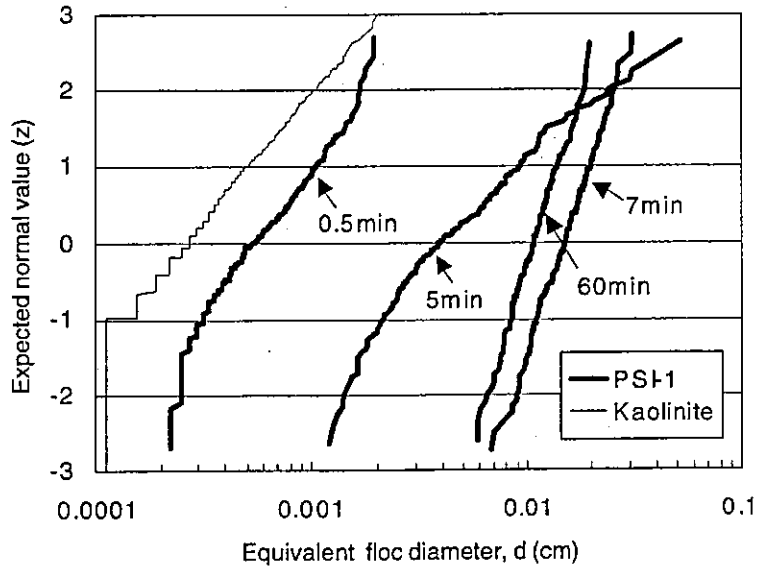

Figure 4 Floc size distribution at each stirring time (PSI-1)

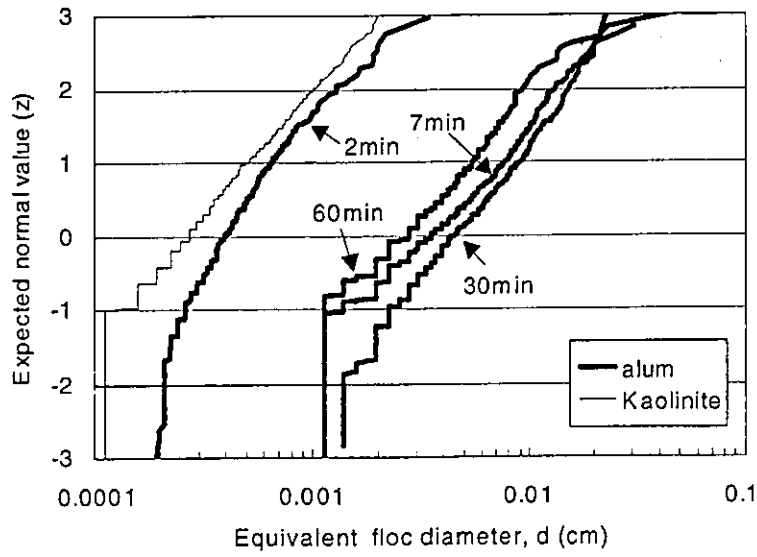

Figure 5 Floc size distribution at each stirring time (alum)

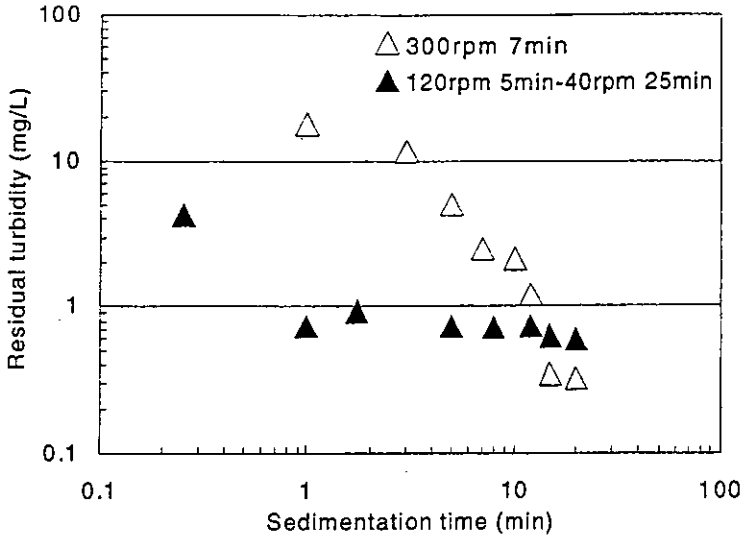

Figure 6 Turbidity removal at different flocculation conditions (PSI-1)

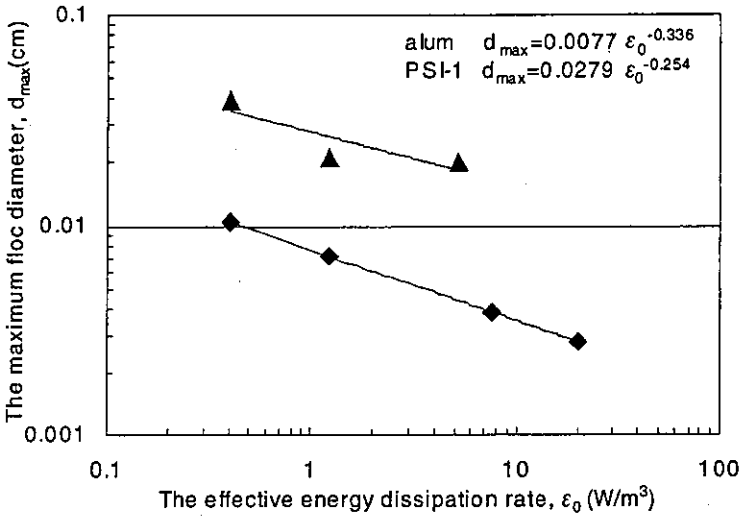

Figure 7 Relationship between the maximum floc diameter and the effective energy dissipation rate

$\varepsilon_0$and the stirring speed, s (rpm) was examined since this is specific to the apparatus. For the authors' apparatus, it was roughly estimated as equation (3).

$$\varepsilon_0 \approx s^{2.5} \times 10^{-6} \tag{3}$$

Also, floc size distributions were measured for each stirring speed designated (for PSI-1: 120, 300 and 500 rpm and for alum: 120, 300, 600 and 800 rpm). The 90%-value of the floc size distribution when the best-grown floc distribution was observed was allocated to d_{max}. As shown in Figure 7, PSI-1 produces larger flocs than alum at the same energy dissipation rate. Tambo and Hozumi (1979) proposed the relative floc binding strength expressed as equation (4).

$$\frac{\sigma_2}{\sigma_1} = \left(\frac{a_2}{a_1}\right)^{-2/3} \left[\frac{(d_{max-2}/1)^{3+K_2}}{(d_{max-1}/1)^{3+K_1}}\right]^{2/3} \tag{4}$$

where (σ_2/σ_1): the relative floc binding strength, 1: a unit to adjust dimension (cm) and the suffixes 1 and 2 denote the characteristic values of floc 1 and 2, respectively.

Here, alum and PSI-1 are allocated to floc 1 and 2, respectively, and ε_0 is estimated to 1.47, which is equivalent to the stirring speed at 300 rpm in the authors' apparatus. Substituting a, K (Figure 2) and d_{max} (Figure 7) to equation (4), $\sigma_{PSI-1}/\sigma_{alum}$ is estimated as

25.3. Tambo *et al.* (1970) estimated the relative floc binding strength of PACl to alum and obtained 2.74. Since these are the results obtained from different coagulation conditions, they cannot be compared quantitatively. But, roughly speaking, it is suggested that PSI-1 produce much stronger flocs than PACl.

Parameters required for the flocculator design

Tambo and Watanabe proposed three parameters required for the flocculator design procedure (Tambo, 1991). These are: m, dimensionless flocculation time; S_m, normalized maximum floc volume (non-dimensional); and K, a coefficient of the floc density function (non-dimensional). The K value for PSI-1 was discussed above and estimated as 1.02, for any FeT ratio (Figure 2). Parameters m and S_m are defined as equations (5) and (6) (Tambo, 1991).

$$m = 1.22\sqrt{\frac{\varepsilon_0}{\mu'}}d_1^3 n_0 t \tag{5}$$

$$S_m = \left(\frac{d_{\max}}{d_1}\right)^3 \tag{6}$$

where μ': viscosity of water (Pa·s), d_1:diameter of initial particles (cm), n_0:number concentration of initial particles (cm^{-3}) and t: flocculation time (s).

Supposing the initial particles of kaolinite clay have the density of 2.6 g/cm^3 and the diameter of 2.5×10^{-4} cm, $n_0 = 2.35 \times 10^6$ cm^{-3} is given when the initial concentration of kaolinite clay is 50 mg/L. Thus, applying equation (5) and (6) to the plots on Figure 7, relationship between S_m and m can be drawn as Figure 8. As far as following the line segments in this figure, the best flocculation is expected. In this occasion, it can be also expected that the number of smaller flocs has decreased. Accordingly, this figure can be utilized to obtain m from S_m in flocculator designing.

Practical design of flocculator

By applying the results yielded above, desirable effective energy dissipation rate and stirring time are to be estimated if the initial particles condition and design condition, in this example, the settling velocity of the maximally grown floc V_{max} (cm/s) are set.

Setting the initial condition of the diameter of initial particles, $d_1 = 2.5 \times 10^{-4}$ cm and the number concentration of them $n_0 = 2.35 \times 10^6$ cm^{-3} (50 mg/L as initial kaolinite clay particles) and design condition $V_{max} = 0.35$ cm/s, the maximum floc size d_{max} is given as

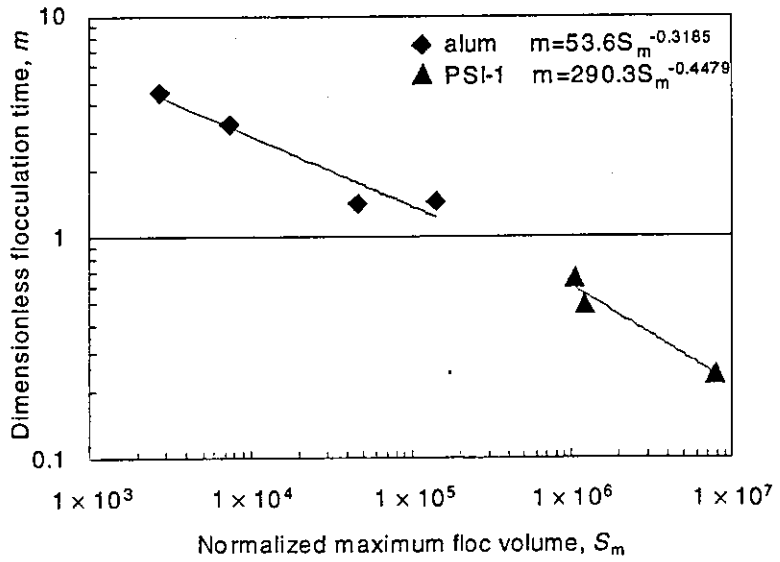

Figure 8 Relationship between S_m and m

Table 1 Examples of design parameters

V_{max} [cm/s]	0.35	0.4	0.5	0.6	0.7
d_{max} [cm]	0.026	0.030	0.037	0.045	0.053
ε_0 [W/m^3]	1.32	0.77	0.32	0.15	0.082
(Stirring speed [rpm])*	(287)	(232)	(162)	(120)	(94)
t [min]	5.8	6.3	7.3	8.2	9.0

* In the case of authors' apparatus.

2.6×10^{-2} cm by applying equation (1) and Figure 3. Then from Figure 7, the effective energy dissipation rate to let the floc grown to this size is calculated to be 1.32 W/m^3, approximately 290 rpm in the authors' apparatus. In this way, normalized maximum floc volume, S_m and dimensionless flocculation time, m are determined as 1.1×10^6 and 0.57, respectively. Finally, the stirring time is estimated as $t = 347$ (s) = 5.8 (min) applying equation (5). According to this estimation, it is suggested that slow mixing process, which is essential for the conventional water purification system using aluminium coagulants, can be eliminated by using PSI. It might lead to diminish the required size of the water purification plant and be useful to construct or renovate the plant in densely populated areas, where open space is scarce. On the contrary, the flocculator proposed above increases the energy consumption rate. Still it is not obvious whether it really consumes more power in full-scaled equipment and it should be examined by pilot plant study or some other way.

The design parameters calculated under various design condition V_{max}, are shown in Table 1. When V_{max} increases, the size required for the sedimentation tank and the energy consumption rate may decrease, on the other hand, the size of flocculator increases. Minimum size of flocculator with sedimentation tank is not discussed in this paper, where flocculation process is especially focused on and the design of sedimentation tank is not considered. Future research is necessary for the optimal design of the rapid sand filtration system.

Conclusions

Physical characteristics of flocs formed by new polymeric inorganic coagulant, PSI, were investigated in this paper and the following results were obtained.

1. The floc density function proposed by Tambo and Watanabe could be also applied to the ferric coagulants, ferric chloride and PSI. Flocs formed by ferric coagulants showed higher floc density than those formed by alum.
2. Either FeT ratio, pH, or rapid mixing speed had little effect on floc density of the flocs formed by PSI.
3. PSI could produce well grown flocs at the stirring speed of 300 rpm. Stirring at 300 rpm for 7 minutes, residual turbidity after 15 minute sedimentation became lower than rapid mixing at 120 rpm for 5 minutes followed by slow mixing at 40 rpm for 25 minutes.
4. Flocs formed by PSI was much stronger than those formed by alum.

Using the data obtained in the experiments, practical design of flocculator was estimated. According to the estimation, it was suggested that rapid mixing process alone can produce well grown flocs and slow mixing process can be eliminated by use of PSI. It might lead to diminish the required size of the water purification plant. PSI coagulant has very much different characteristics from aluminium coagulants and should be evaluated under appropriate operating condition for PSI.

Acknowledgement

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References

Dolejs, P. (1993). Influence of algae and their exudates on removal of humic substances and optimal dose of coagulant. *Wat. Sci. Tech.*, **27**(11), 123–132.

Ebie, K., Azuma, Y., Asaka, H., Yamaki, A. and Hagishita, T. (2001). Basic study on the optimization of the stirring condition in coagulation process. *Proc. 52nd JWWA research conference and symposium*, 96–97, Japan Water Works Association. (in Japanese)

Gregor, J.E., Fenton, E., Brolenshire, G., Van den Brink, P. and O'Sullivan, B. (1996). Interactions of calcium and aluminium ions with alginate. *Wat. Res.*, **30**, 1319–1324.

Hasegawa, T., Wang, J., Ehara, Y., Kurokawa, M., Hashimoto, K. and Okada, M. (2000). Removal of algae and DOC by polysilicato-iron coagulant. *Proc. 12th IWA-ASPAC Regional Conference and Exhibition*, 106–112.

Hashimoto, K., Hasegawa, T., Wang, J., Kurokawa, M. and Watanabe, Y. (1997). Separation of *Microcystis sp.* by polysilicato-iron coagulant. *Proc. 6th Korea/Japan Symposium on Water Environment*, 129–136.

Magara, Y., Tanaka, K., Saito, S., Shioda, M. and Terada, S. (1992). *Investigation of actual conditions regarding the interference caused by organisms in drinking water, Study report on the interference of coagulation by algae.* Japan Water Works Association, Tokyo, Japan, pp. 8–29. (in Japanese)

Tambo, N., Yamada, K. and Hozumi, H. (1970). Study on strength of floc. *J. Japan Water Works Association*, **427**, 4–15. (in Japanese)

Tambo, N. and Watanabe, Y. (1979). Physical characteristics of flocs I, The floc density function and aluminium floc. *Wat. Res.*, **13**, 409–419.

Tambo, N. and Hozumi, H. (1979). Physical characteristics of flocs II, Strength of floc. *Wat. Res.*, **13**, 421–427.

Tambo, N. (1991). Criteria for flocculator design. *J. Water SRT-Aqua*, **40**(2), 97–102.

Wang, J., Deevanhxay, P., Hasegawa, T., Ehara, Y., Kurokawa, M., Hashimoto, K., Nishijima, W. and Okada, M. (2002). A pilot plant study of polysilicato-iron coagulant. *Wat. Sci. Tech.: Wat. Suppl.*, **2**(2), 107–113.

World Health Organization (1998). *Guidelines for drinking-water quality, Second edition, Addendum to volume 1 recommendations* pp. 3–4. World Health Organization, Geneva, Switzerland.

Study of 1,4-Dioxane Intake in the Total Diet Using the Market-Basket Method

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1,4-Dioxane has been classified by the US Environmental Protection Agency and the International Agency for Research on Cancer as a compound that may be carcinogenic in humans. Although there are several reports of 1,4-dioxane being detected in the environment, such as in tap water, there have been few reports on the content of 1,4-dioxane in food. We therefore studied the intake of 1,4-dioxane in food based on the average intake of food in the Kanto area of Japan as reported by the Ministry of Health, Labor and Welfare. The food was cooked in the normal manner and then homogenized in a mixer. A 20 g of sample of the homogenate was added to a solution of the purified water with 0.2 μg of 1,4-dioxane-d_8 as a surrogate and the 200 ml azeotropic solution was recovered using the steam distillation method. This solution was applied to a pair of active carbon solid-phase cartridges and the analyte was eluted from each cartridge with dichloromethane. The eluted solution was prepared for gas chromatographic/mass spectrometric analysis by reduction to a volume of 1 ml under a gentle stream of nitrogen. The detection limit of the analysis was 2 μg/kg. We found that the 1,4-dioxane content of 12 food groups ranged between 2 μg/kg and 15 μg/kg. From these results, the total daily intake of 1,4-dioxane was calculated to be 0.440 μg. An intake of this magnitude corresponds to 0.055% of the calculated total daily intake (TDI) (16 μg/kg body weight/day). This study indicates that the amount of 1,4-dioxane intake contributed by food is very low and that this value does not represent a potential problem as it does not raise the risk of carcinogenesis.

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Key words —— 1,4-dioxane, total diet, risk

INTRODUCTION

1,4-Dioxane is used extensively as an industrial solvent in dyes, paints, lacquers, varnishes, oils, waxes, and resins and is also added as a stabilizer to chlorinated solvents.[1] 1,4-Dioxane is highly soluble in water, forming an azeotropic mixture, and when discharged into the atmosphere it returns to the surface as rainwater. As a property of its low adsorption to soil, 1,4-dioxane then permeates into the groundwater and causes water pollution over the long term. Therefore 1,4-dioxane has the potential to cause widespread contamination of the environment. There are reports of 1,4-dioxane being detected in river water at levels between 0.1 and 16.0 mg/l, and in groundwater at a maximal concentration of 94.8 mg/l in Japan.[2] Several other studies have also shown high levels of 1,4-dioxane pollution in groundwater.[2–4] As the removal of 1,4-dioxane in water purification systems is difficult, these findings raise concerns regarding chronic exposure to 1,4-dioxane in drinking water.

1,4-Dioxane has been classified as a carcinogenic compound by both the USA Environmental Protection Agency[5] and the International Agency for Research on Cancer (IARC).[6] Long-term oral administration of 1,4-dioxane has been shown to cause tumors in the liver and gallbladder in guinea pigs,[7] and in the nasal cavity and liver of rats.[8–11] 1,4-Dioxane has also demonstrated promoter activity in studies in mice using a two-stage carcinogenic test.[12]

Levels of 1,4-dioxane between 0.2 mg/l and 1.5 mg/l were also detected in tap water samples collected during 1995 and 1996 from six cities in Kanagawa prefecture, Japan.[13] This finding raises the possibility that food may also have become con-

taminated. Although 1,4-dioxane is now included in the quality standards for drinking water in Japan, there have been few reports on the contents and intake of 1,4-dioxane in food. To safeguard human health, it is important to determine the Japanese intake levels of 1,4-dioxane through food. This paper describes a study on the intake of 1,4-dioxane through food in Japan using the market-basket method.

MATERIALS AND METHODS

Chemicals —— 1,4-Dioxane was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), 1,4-dioxane-d_8 from Sigma-Aldrich Co. Ltd. (St. Louis, MO, U.S.A.), dichloromethane from Kanto Chemical Industry Co. Ltd. (Tokyo, Japan), ethanol from Katayama Chemical Industry Co. Ltd. (Osaka, Japan), acetonitrile and acetone from Wako Chemical Industry Co. Ltd. (Osaka, Japan), and the antifoaming agent silicon TAS730 from Toshiba Silicon Co. Ltd. (Tokyo, Japan). All solvents were of the highest reagent grade. Purified water was prepared using a Milli-Q water purification PSS20 system (Millipore Corp., Bedford, MA, U.S.A.).

Preparation of Standard Solutions —— A stock solution of 1,4-dioxane (1 mg/ml) was prepared in dichloromethane, and a stock solution of 1,4-dioxane-d_8 (10 mg/ml) was prepared in ethanol. The stock solution of 1,4-dioxane-d_8 was then diluted with dichloromethane to a final concentration of 40 μg/ml. The 1,4-dioxane stock solution was diluted with dichloromethane and used to prepare the working standard solutions containing 1,4-dioxane-d_8 0.2 μg/ml in the concentration range of 0.04–1 μg/ml. A solution of 1,4-dioxane-d_8 2 μg/ml was prepared in acetonitrile and was added to samples to determine the recovery rate of the analytical procedure.

Preparation of Food Samples —— The food ingredients were purchased at a general market in Setagaya-ku, Tokyo, Japan in April and May 2000. The quantity of each food item was determined on the basis of the results of the average intake of food in the Kanto area reported by the Ministry of Health, Labor and Welfare (Table 1), using six times the weight of each food group for preparation. Food was cooked in a manner similar to that used in normal homes and was then homogenized in a mixer (Hamilton Beach/Proctor-Silex, Inc., Washington, NC, U.S.A., Model 911). Food that was difficult to homogenize was made uniform by the addition of purified water. In the preparation and cooking of the food, utensils made from wood, aluminum, fluororesin iron, and plastic were used and included items such as a chopping block, pots, and pans. These utensils were washed in a manner similar to that used in normal homes, and tap water was used for cooking. The food homogenates of each group were stored in glass bottles with silicon seals and were kept frozen at –20°C until analyzed.

Extraction of 1,4-Dioxane —— A 20 g of sample of each homogenate was placed in a 500 ml eggplant-type flask, followed by the addition of 150 ml of purified water and 100 μl of 2 μg/ml 1,4-dioxane-d_8 solution. Two hundred milliliters of aqueous solution was recovered from the sample mixture using the steam distillation method. This solution was then passed through a pair of active carbon solid-phase cartridges equilibrated with 20 ml of dichloromethane, 30 ml of acetone, and then 40 ml of purified water. Extraction of the water samples was carried out at a flow rate of 10 ml/min. After the water sample had passed through the cartridges, the cartridges were washed with 5 ml of purified water at the same flow rate. Dried nitrogen gas was then passed through the cartridges for 30 min, and the analyte was eluted from the each cartridge, with the cartridge for the water adsorption attached at the bottom with dichloromethane 3 ml. The cartridge for water adsorption was washed with dichloromethane 20 ml prior to use. The eluted solution was then reduced to a volume of 1 ml under a gentle stream of nitrogen for gas chromatographic/mass spectrometric (GC/MS) analysis.

GC/MS Analysis —— An Agilent 6890/5973N (Agilent Technologies Inc., Palo Alto, CA, U.S.A.) instrument was used for the GC/MS analysis, with separation carried out on a SPB-624 capillary column (60 m × 0.25 mm i.d. × 1.4-μm film thickness) (Sigma-Aldrich Co. Ltd.). Helium was used as the carrier gas with a column flow rate of 1 ml/min in the constant flow mode. The column temperature was kept at 60°C for 1 min, then programmed to increase by 5°C per minute to 130°C and then 20°C per minute to 230°C. Pulsed splitless injection was used with a pulse pressure of 400 kPa (1 min). The ion source temperature was kept at 230°C with the mass spectrometer operated in the EI mode. In the selected ion monitoring (SIM) mode, the monitoring ions were 58 and 88 for 1,4-dioxane and 64 and 96 for 1,4-dioxane-d_8. The injection volume was 2.0 μl. A calibration curve was prepared from the ratio of the peak height of 1,4-dioxane and 1,4-di-

Table 1. List of Food in the Total Diet Study

Group	Food Group	Food	Daily Intake (g)
I	Rice	Rice	149.0
	Rice products	Rice vermicelli	4.1
II	Barley	Oatmeal	0.2
	Flour	Wheak flour	8.3
	Bread	Bread	36.1
	Sweet bun	Bean-jam bun	8.6
	Noodles	Japanese wheat noodles	39.2
	Noodles, macaroni	Buckwheat	6.5
	Instant noodles	Instant noodles	3.7
	Grain	Cornflakes	2.5
	Seed	Crushed almonds	2.0
	Sweet potato	Sweet potato	8.6
	Potato	Potato	35.9
	Tubers and roots	Taro	10.4
	Product of tubers and roots	Konjak	13.5
III	Sugar	Granulated sugar	7.6
	Jam	Strawberry jam	1.6
	Candy	Caramel	0.3
	Rice cracker	Rice cracker	2.0
	Cake	Pound cake	4.0
	Biscuit	Biscuit	3.3
	Other snacks	Japanese fried-dough cookies	15.0
		Azuki bean jelly	
		Chocolate	
IV	Butter	Butter	1.3
	Margarine	Margarine	1.7
	Vegetable oil	Soybean oil	10.4
	Animal oil	Lard	0.2
	Mayonnaise	Dressing	5.7
V	Soybean paste	Soybean paste	12.8
	Beancurd (tofu)	Beancurd (tofu)	35.4
	Product of beans	Deep-fried beancurd	6.0
	Soybean products	Freeze-dried beancurd	9.9
	Beans		2.6
VI	Citrus	Navel orange	30.6
	Apple	Apple	26.6
	Banana	Banana	7.7
	Strawberry	Strawberry	0.2
	Fruit	Watermelon	49.0
		Loquat	
		Japanese apricot	
	Juice	Tomato juice	17.0
VII	Carrot	Carrot	24.0
	Spinach	Spinach	19.7
	Green pepper	Green pepper	4.7
	Tomato	Tomato	21.7
	Green and yellow vegetables	Broccoli	30.8
		Celery	
		Okra	

Table 1. Continued

Group	Food Group	Food	Daily Intake (g)
VIII	Japanese radish (daikon)	Japanese radish	36.0
	Onion	Onion	27.7
	Cabbage	Cabbage	24.8
	Cucumber	Cucumber	14.7
	Napa cabbage	Napa cabbage	18.2
	Vegetables	Burdock (gobo root)	44.5
		Beansprouts	
		Eggplant	
	Pickles	Pickles (nozawa-na)	6.5
	Pickled Japanese radish	Fukujinn-zuke	15.5
	Mushrooms	Mushrooms	12.8
	Seaweeds	Green laver	5.6
IX	Soy sauce	Soy sauce	19.9
	Sauces	Ketchup	5.4
	Salt	Salt	1.3
	Seasoning	Sauce	11.6
	Sake	Sake	15.5
	Beer	Beer	64.2
	Liquor	Wine	12.4
	Soft drinks	Soft drinks	69.4
		Tea	
X	Salmon and trout	Salmon	3.3
	Tuna	Tuna	8.8
	Bream and flatfish	Flatfish	8.0
	Horse mackerel and sardine	Horse mackerel	12.5
	Raw fish	Ayu	6.4
		Kisu	
		Halfbeak	
	Cuttlefish, octopus, and crab	Octopus	13.6
	Shellfish	Scallop	4.7
	Salted fish	Salted cod	8.7
	Dried fish	Dried sardine	9.5
	Canned fish	Bonito	2.9
	Cooked fish	Smelt	0.7
	Cooked fish paste	Hannpen	12.2
	Fish product	Fish sausage	0.3
XI	Beef	Beef	21.7
	Pork	Pork	31.9
	Chicken	Chicken	19.8
	Whale		0.0
	Other animal meat	Lamb	0.9
	Ham and sausage	Pork loin ham	11.0
	Eggs	Chicken eggs	38.3
XII	Milk	Milk	116.4
	Cheese	Cheese	2.8
	Daiury products	Yoghurt	21.1
	Others	Sake sediment	5.8

oxane-d_8. Quantitative analysis of the food samples was carried out using methodology identical to that used in the preparation of the calibration curve.[13]

RESULTS AND DISCUSSION

Detection Limit in Food Samples

The minimum detection level of 1,4-dioxane-d_8 added as an internal standard was 0.04 μg/l (S/N = 10). The minimum detection limit of 1,4-dioxane in the prepared food was calculated to be 2 μg/kg using the following formula: (0.04 μg/l × 1 ml)/20 g = 0.002 μg/g = 2 μg/kg , in which 1 ml indicates the final volume for GC/MS analysis and 20 g indicates the weight of the food homogenate.

Recovery Test of 1,4-Dioxane

The concentration of 1,4-dioxane in the purified water and tap water used in the analysis and in the preparation of the food samples was less than 0.04 μg/ml. This level represented the minimum detection limit when the analysis was carried out in the manner used for the food samples.

After the addition of 1,4-dioxane 0.2 μg and 1,4-dioxane-d_8 1 μg to 4 g of the prepared food samples, the recovery rate of 1,4-dioxane was obtained using the method described in the MATERIALS AND METHODS section. Table 2 shows that the recovery rate was between 99% and 111% in the 12 groups. These results indicate there was no problem with the efficiency of extraction when 1,4-dioxane was added to the food samples at a concentration < 0.2 μg/4 g (50 μg/kg).

Content of 1,4-Dioxane in the Food Samples

The extraction of 1,4-dioxane from each 20 g prepared food sample was carried out according to the method described in the MATERIALS AND METHODS section. Table 3 shows that the content of 1,4-dioxane in the 12 food groups was between 2 μg/kg, the detection limit of the analysis, and 15 μg/kg. If the food sample was difficult to homogenize after cooking, an appropriate quantity of purified water was added to achieve homogeneity (*i.e.*, groups I, II, III, and X in Table 4). The weight of the food used for the extraction of 1,4-dioxane before and after cooking was then calculated.

The intake of 1,4-dioxane was calculated based on the average intake of food in the Kanto area as reported by the Ministry of Health, Labor and Welfare. For example, the calculation in group II included a food sample of 1061.6 g that was added to 600.0 g of purified water for cooking and then homogenized. The actual weight of food for extraction was 18.34 g calculated as (20 g/1157.5 g) × (1061.6 g + 600.0 g) × {1061.6 g/(1061.6 g + 600.0 g)}. Since the intake of group II food was 175.5 g, the intake of 1,4-dioxane from food in this group was 0.057 μg calculated as (175.5 g/18.34 g) × 6 μg/kg × (1/1000). From the results of the content of each group in Table 4, the daily total intake of 1,4-dioxane from food was calculated to be 0.440 μg.

Table 2. Recovery Rate of 1,4-Dioxane Added to Food Samples

Group	Recovery Rate (%)
I	104
II	101
III	105
IV	101
V	106
VI	100
VII	100
VIII	100
IX	99
X	102
XI	104
XII	111

Table 3. Content of 1,4-Dioxane in Food Samples

Group	Content (mg/kg)
I	ND
II	6
III	6
IV	8
V	3
VI	4
VII	3
VIII	8
IX	7
X	5
XI	6
XII	13

ND: not detectable.

Risk from 1,4-Dioxane in Food

There is evidence that long-term oral administration of 1,4-dioxane causes hepatic and nasal cavity tumors in rodents,[8–12] and accordingly the IARC has classified 1,4-dioxane as a group 2B carcino-

Table 4. Intake of 1,4-Dioxane from Food

Group	Weight before Cooking (g)	Weight of Added Water (g)	Weight after Cooking (g)	Actual Weight of Food for Extraction (g)[a]	Intake of Food (g)	Content in Food (μg/kg)	Intake of 1,4-Dioxane (μg)
I	894.0	1143.3	1715.0	10.43	153.1	ND	0.000
II	1061.6	600.0	1157.5	18.34	175.5	6	0.057
III	202.8	100.0	202.8	20.00	33.8	6	0.010
IV	1114.0		1114.0	20.00	19.3	8	0.008
V	727.9		400.2	36.38	66.7	3	0.006
VI	981.5		981.5	20.00	131.1	4	0.026
VII	605.4		609.0	19.88	100.9	3	0.015
VIII	1237.8		1143.7	21.65	206.3	8	0.076
IX	416.4		416.4	20.00	199.7	7	0.070
X	550.2	300.0	524.0	21.00	91.6	5	0.022
XI	608.9		908.9	13.40	123.6	6	0.055
XII	1019.4		1019.4	20.00	146.1	13	0.095

a) 1 : 20 g was used for extraction.

gen.[6] With regard to a cancer endpoint, a total daily intake (TDI) of 16 μg of 1,4-dioxane/kg body weight/day has been calculated by applying an uncertainty factor of 1000 that incorporates 100 for inter- and intraspecies variation and 10 for nongenotoxic carcinogenicity to the no observed adverse effect level of 16 μg/kg body weight/day, as found in a long-term study involving drinking water in rats.[14,15] The 0.440 μg intake of 1,4-dioxane we measured in our study corresponds to 0.055% of the calculated TDI (0.440 μg/{16 μg/kg body weight/day × 50 kg}). We therefore conclude that the intake of 1,4-dioxane from food appears to be very low and that this value does not increase the risk of carcinogenicities.

Acknowledgements We thank Mrs. Emi Hayabe-Hirakawa for help in the preparation of the food samples. This work was supported by Grants-in-Aid from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1) Budavari, S. (Ed.) (1996) *The Merk Index*, 12 th edn., Merk & Co. Inc., Whitehouse Station, NJ, p. 3358.
2) Abe, A. (1999) Distribution of 1,4-dioxane in relation to possible sources in the water environment. *Sci. Total Environ.*, **227**, 41–47.
3) Ministry of the Environment (Planning and Coordination Bureau S) (1998) *Outline of 1998 study results on designated chemicals*, pp. 26–37.
4) Magara, Y., Sudo, R., Nakamuro, K., Yuasa, A., Abe, A., Sato, C., Sekine, H., Oosawa, E., Terasawa, K., Kawachi, T., Shiozaki, J., Aizawa, T., Ando, M. and Nishimura, T. (1998) Comprehensive approaches to the management of surfactants and related chemicals in water sources and drinking water treatment processes. In *Environmental Research in Japan, 1998* (National Resarch Institutes of Government Ministries and Agencies, Eds.), No. 1, pp. 8.1–8.33.
5) US EPA (1993) *Integrated Risk Information System (IRIS) on 1,4-Dioxane. Environmental Criteria and Assessment Office* (Office of Research and Development, Eds.), Cincinnati, OH.
6) IARC (1999) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 71, Pt 2, *Re-evaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide*, World Health Organization, Lyon, pp. 589–602.
7) Hoch-Ligeti, C. and Argus, M. F. (1970) Effect of carcinogens on the lung of guinea pigs. Morphology of experimental respiratory carcinogenesis. In *Publication No. CONF-700401* (Nettesheim, P., Hanna, M. G., Jr. and Deatherage, J. W., Jr., Eds.), Technical Information Service, U.S. Department of Commerce, Springfield, VA, pp. 267–279.
8) Argus, M. F., Arcos, J. C. and Hoch-Ligeti, C. (1965) Studies on the carcinogenic activity of protein-denaturing agents: hepatocarcinogenicity of dioxane. *J. Natl. Cancer Inst.*, **35**, 949–958.
9) Argus, M. F., Sohal, R. S., Bryant, G. M. and Hoch-Ligeti, C. (1973) Dose-response and ultrastructural alterations in dioxane carcinogenesis. Influence of methylchlolanthrene on acute toxicity. *Eur. J. Cancer*, **9**, 237–243.
10) Hoch-Ligeti, C., Argus, M. F. and Arcos, J. C. (1970) Induction of carcinomas in the nasal cavity of rats

by dioxane. *Br. J. Cancer*, **24**, 165–167.
11) Kociba, R. J., McCollister, S. B., Park, C., Torkelson, T. R. and Gehring, P. J. (1974) 1,4-Dioxane 1. Results of a 2-year ingestion study in rats. *Toxicol. Appl. Pharmacol.*, **30**, 275–286.
12) King, M. E., Shefiner, A. M. and Bates, R. R. (1973) Carcinogenesis bioassay of chlorinated dibenzodioxins and related chemicals. *Environ. Health Prespect*, **5**, 163–170.
13) Abe, A. (1997) Determination method for 1,4-dioxane in water samples by solid phase extraction-GC/MS. *J. Environ. Chem.*, **7**, 95–100 (in Japanese).
14) Kochiba, R. J., McCollister, S. B., Park, C., Torkelson, T. R. and Gehring, P. J. (1974) 1,4-Dioxane. I. Results of a 2-year ingestion study in rats. *Toxicol. Appl. Pharmacol.*, **60**, 287–300.
15) World Health Organization (Ed.), *WHO Guideline for Drinking-water Quality*, 3rd. edn., World Health Organization, (in press).

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3) Meister, B., Revel, T. M. and Caprio, M. N. (1999) Molecular mechanism of cell death. In *Handbook of Health Science* (Clarke, A. and Young, T. A., Eds.), Carrie Press, Tokyo, pp.135–137.

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Dose-related changes of oxidative stress and cell proliferation in kidneys of male and female F344 rats exposed to potassium bromate

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It is still of importance to investigate renal carcinogenesis by potassium bromate ($KBrO_3$), a by-product of water disinfection by ozonation, for assessment of the risk to man. Five female F344 rats in each group were given $KBrO_3$ at a dose of 300 mg/kg by single i.g. intubation or at a dose of 80 mg/kg by single i.p. injection, and were killed 48 h after the administration for measurements of thiobarbituric acid-reactive substances (TBARS) and 8-oxodeoxyguanosine (8-oxodG) levels in the kidney. Both levels in the treated animals were significantly elevated as compared with the control values. In a second experiment, 5 male and female F344 rats in each group were administered $KBrO_3$ at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm in the drinking water for 4 weeks. $KBrO_3$ in the drinking water did not elevate TBARS in either sex at any of the doses examined, but 8-oxodG formation in both sexes at 250 ppm and above was significantly higher than in the controls. Additionally, the bromodeoxyuridine-labeling index for proximal convoluted tubules was significantly increased at 30 ppm and above in the males, and at 250 ppm and above in the females. α2u-Globulin accumulation in the kidneys of male rats was increased with statistical significance at 125 ppm and above. These findings suggest that DNA oxidation induced by $KBrO_3$ may occur independently of lipid peroxidation and more than 250 ppm $KBrO_3$ in the drinking water can exert a carcinogenic effect by way of oxidative stress. (Cancer Sci 2004; 95: 393–398)

Potassium bromate ($KBrO_3$) was at one time widely used as a maturing agent for flour and as a dough conditioner.[1] It was, however, demonstrated to induce renal cell tumors in male and female F344 rats after oral administration for 2 years in the drinking water[2] and the use of $KBrO_3$ as a food additive is now limited or prohibited, so that exposure of humans via food is very low.[3] Nevertheless, there is still concern regarding this chemical in the environment. In order to avoid the formation of trihalomethanes, major by-products in the process of drinking water chlorination[4] that are carcinogenic in rodents,[5] ozone disinfection has been proposed as an alternative method.[6] However, it has been shown that ozonation of surface water can generate bromate as one of various by-products in treated drinking water,[7] implying a potential hazard.

$KBrO_3$ has been classified as a genotoxic carcinogen based on positive mutagenicity in the Ames,[8] chromosome aberration[9] and micronucleus tests.[10] It has the potential to induce 8-oxodeoxyguanosine (8-oxodG) formation both *in vitro* and *in vivo*,[11–14] and since ribo- and deoxyribonucleosides of 8-oxodG induce sister chromatid exchange in human lymphocytes[15] and 8-oxodG pairs with adenine as well as cytosine, generating GC-to-TA transversion upon replication by DNA polymerases,[16] it has been postulated that this oxidized base is responsible for the mutagenicity and carcinogenicity.[17,18] The formation of oxidized base also indicates that the intra-nuclear redox status is altered in an oxidative direction, and this may lead to the induction of aberrant transcriptional events. However, except for our previous paper,[19] we know of no data showing a direct correlation between actual carcinogenic doses and 8-oxodG formation in kidney DNA. Likewise, although it has been proposed that reactive free radicals resulting from the oxidizing property of $KBrO_3$ also attack membrane lipids to induce cellular lipid peroxidation (LPO) in male rats,[20,21] it remains uncertain whether LPO indeed occurs concomitantly with DNA oxidation during carcinogenesis. In view of the possible role of various reactive aldehydes as end products of LPO in tumorigenesis,[22,23] it is necessary to assess their participation in $KBrO_3$ carcinogenesis.

A two-stage model using *N*-ethyl-*N*-hydroxyethyl-nitrosamine (EHEN) as an initiator has supplied clear evidence that $KBrO_3$ has promoting activity for renal carcinogenesis in male and female rats.[24,25] We have also shown that numbers of bromodeoxyuridine (BrdU)-incorporating cells in kidney tubules are elevated in male and female rats exposed to $KBrO_3$ at a dose of 500 ppm in the drinking water.[19,26] While we have hypothesized the involvement of oxidative stress induced by $KBrO_3$ in the promoting activity, our previous data also suggest that the promoting action observed in male rats may be dependent on cell proliferation due to accumulation of α2u-globulin, a male rat specific urinary protein.[26] Elimination of this possibility as a factor contributing to $KBrO_3$ promoting activity is a prerequisite for accurate assessment of the carcinogenic risk in humans.

In the present study, in order to confirm a positive correlation between oxidized DNA base formation and occurrence of LPO, we measured the levels of 8-oxodG and thiobarbituric acid-reactive substances (TBARS) in kidneys of F344 female rats given $KBrO_3$ by single administration at high doses. Secondly, we examined the dose-response effects with reference to 8-oxodG levels, TBARS, BrdU-labeling and α2u-globulin accumulation in kidney, as well as serum creatinine (CRN) level, of male and female rats, employing the same doses and route as used in the previous carcinogenicity tests and promoting activity assays. The aim was to clarify the possibility that LPO and oxidative DNA damage participate in $KBrO_3$ initiation and to cast light on the effects of oxidative stress in the promotion phase.

Materials and Methods

Chemicals. $KBrO_3$ was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Alkaline phosphatase and BrdU were obtained from Sigma Chemical Co. (St. Louis, MO) and nuclease P1 was from Yamasa Shoyu Co. (Chiba).

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Animals, diet and housing conditions. The protocols for this study were approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five-week-old male and female F344 rats (specific pathogen-free) were purchased from Charles River Japan (Kanagawa) and housed in polycarbonate cages (5 rats per cage) with hardwood chips for bedding in a conventional animal facility maintained under conditions of controlled temperature (23±2°C), humidity (55±5%), air change (12 times per h) and lighting (12 h light/dark cycle). The animals were given free access to CRF-1 basal diet (Charles River Japan) and tap water, and were used after a 1-week acclimation period.

Animal treatments.

Experiment I: Five female rats in each group were given $KBrO_3$ at a single dose of 300 mg/kg by i.g. administration or 80 mg/kg by i.p. injection. Control animals received saline at the same volume as the i.p. administration group. All animals were killed 48 h after the administration under ethyl ether anesthesia, and the right and half of the left kidneys were immediately removed and frozen with liquid nitrogen and stored at −80°C until measurement of 8-oxodG in nuclear DNA and TBARS levels. The remaining kidney tissue was fixed in buffered formalin and then routinely processed for embedding in paraffin, sectioning and H&E staining. The doses and experimental period followed reported conditions under which the 8-oxodG and TBARS levels in kidney were significantly increased.[27]

Experiment II: Five male and female rats in each group were administered $KBrO_3$ solution at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm in the drinking water for 4 weeks. All animals were injected with BrdU (100 mg/kg) i.p. twice a day for the final 2 days of the exposure and once on the day of termination, 2 h before killing. For analysis of CRN, the animals were anesthetized with ethyl ether and blood was collected from the aorta. Determination of CRN was carried out at SRL, Inc. (Tokyo). At necropsy, the right kidneys were fixed in ice-cold acetone for 3 days and processed for embedding in paraffin, sectioning (4 μm), and immunostaining for BrdU after histochemical demonstration of γ-glutamyltranspeptidase (γ-GT) activity. The left kidneys were frozen and stored as in Experiment I until measurement of 8-oxodG in nuclear DNA, and TBARS levels and α2u-globulin contents in the homogenates.

Measurement of nuclear 8-oxodG. The 8-oxodG levels in kidney DNA were determined according to the method of Nakae *et al.*[28] Nuclear DNA was extracted with a DNA Extracter WB Kit (Wako Pure Chemical Industries, Ltd., Osaka). The DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase and levels of 8-oxodG (8-oxodG/10^5 deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Coulochem II, ESA, Bedford, MA).

Measurement of TBARS. Malondialdehyde (MDA, nmol/g) was assessed as an index of LPO by the method of Uchiyama and Mihara.[29] A 0.1 g portion of kidney was homogenized with 0.9 ml of 1.15% KCl solution and the TBARS content was measured.

α2u-Globulin content. α2u-Globulin accumulation in kidneys was measured using a commercially available ELISA kit (Quatikinine M, R&D Systems, Inc., MN). Absorbance at 450 nm was determined using a microplate reader (Thermo Labsystems, Vantaa, Finland), with the reference wavelength set at 590 nm.

Immunohistochemical procedures. For immunohistochemical staining of BrdU, sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (Becton Dickinson) (1:100), biotin-labeled horse anti-mouse IgG (1:400) and avidin-biotin-peroxidase complex (ABC) after denaturation of DNA with 4 *N* HCl. Before the denaturation step, sections were processed histochemically for demonstration of γ-GT activity by the method of Rutenburg *et al.*[30] using L-glutamyl-4-methoxy-β-naphthylamide (Polysciences, Ltd., Warrington, PA) as a substrate in order to assist in distinguishing the three kinds of tubules, as previously described.[26] The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). The immunostained sections were lightly counterstained with hematoxylin for microscopic examination.

Cell proliferation quantification. Cells of the three kinds of tubules in the kidney were identified on the basis of γ-GT activity and morphology as previously described.[31] At least 3000 tubular cells in each kidney were counted. The labeling index (LI) was calculated as the percentage of cells positive for BrdU incorporation.

Statistics. The significance of differences in the results from Experiment I was evaluated with Student's *t* test. For Experiment II ANOVA was used, followed by Dunnett's multiple comparison test.

Results

Experiment I. The data for 8-oxodG and TBARS levels in kidneys of female rats given $KBrO_3$ by single administration at doses of 300 mg/kg (i.g.) or 80 mg/kg (i.p.) are summarized in Fig. 1. Values for both parameters were significantly ($P<0.01$) elevated as compared with the controls, in line with previous data. Histopathological examination revealed severe nephrotoxicity characterized by hemorrhage and protein diapedesis in Bowman's capsule, accumulation of hyaline droplet-like material and basophilic alteration in proximal tubules, and extensive necrosis of collecting ducts (Fig. 6, A and B).

Experiment II. As shown in Fig. 2, $KBrO_3$ in the drinking water did not cause elevation of TBARS in kidneys of either sex at any of the doses examined. However, 8-oxodG levels in male

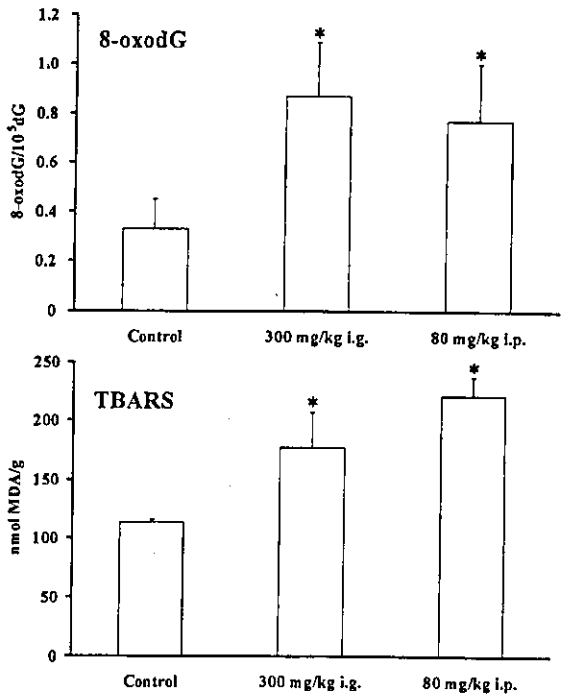

Fig. 1. 8-OxodG and TBARS levels in kidneys of female rats 48 h after single i.g. or i.p. administration of $KBrO_3$ at the dose of 300 or 80 mg/kg, respectively. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group treated with saline alone.

rats exposed to $KBrO_3$ in the drinking water were elevated at concentrations of 250 ppm and above in a clearly dose-dependent manner (250 ppm, $0.57\pm0.19/10^5$ dG, $P<0.01$; 500 ppm, $0.71\pm0.21/10^5$ dG, $P<0.01$) as compared to the control value ($0.31\pm0.06/10^5$ dG). Likewise, 8-oxodG levels in female rats were $0.51\pm0.10/10^5$ dG at 250 ppm and $0.70\pm0.16/10^5$ dG at 500 ppm, which were statistically significantly higher ($P<0.01$) than the control value ($0.25\pm0.05/10^5$ dG). Histopathologically, although degeneration of proximal tubules was dose-dependently observed in the males at 60 ppm and above, there were no overt nephrotoxicity in the females at any of the doses examined. Fig. 3 illustrates changes in BrdU-LI for each tubule type in male and female rats treated with $KBrO_3$ in the drinking water at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm for 4 weeks. BrdU-LIs of proximal convoluted tubular cells (PCT) in the males were elevated in a dose-dependent manner, with significant increases at 30 ppm (1.69±0.32%, $P<0.01$), 60 ppm (2.67±0.45%, $P<0.01$), 125 ppm (4.23±0.80%, $P<0.01$), 250 ppm (6.11±2.23%, $P<0.01$) and 500 ppm (9.10±1.40%, $P<0.01$), as compared to the control value (0.87±0.32%). In the females, although there was no change up to 125 ppm, dose-dependent increase was subsequently observed to 1.29±0.39% at 250 ppm and 2.22±0.37% at 500 ppm, both of which were statistically siginificant ($P<0.01$) as compared to the control value (0.59±0.14%) (Fig. 6C). On the other hand, no change in BrdU-LIs for other tubules was found at any dose in either sex. Fig. 4 summarizes data for α2u-globulin accumulation in kidneys of male and female rats given $KBrO_3$ in the drinking water. In the males, increase was evident at 30 ppm and above in a dose-dependent fashion, the elevation being statistically significant at 125 ppm (1.37±0.18 mg/ml, $P<0.01$), 250 ppm (1.96±0.24 mg/ml, $P<0.01$) and 500 ppm (3.50±0.26 mg/ml, $P<0.01$) as compared to the control value (0.80±0.16 mg/ml). In contrast, α2u-globulin contents in the females were much lower than those in the males and were not changed by $KBrO_3$ exposure. Fig. 5 shows the changes of serum CRN levels in rats of both sexes given $KBrO_3$ in the drinking water. In contrast to the male data, revealing a slight, but statistically significant elevation at 250 ppm and above, there was no change among the female groups.

Discussion

It is generally accepted that oxygen radicals can attack DNA to produce damaged bases, including 8-oxodG, and/or initiate the oxidative decomposition of cellular membranes by LPO,[23]

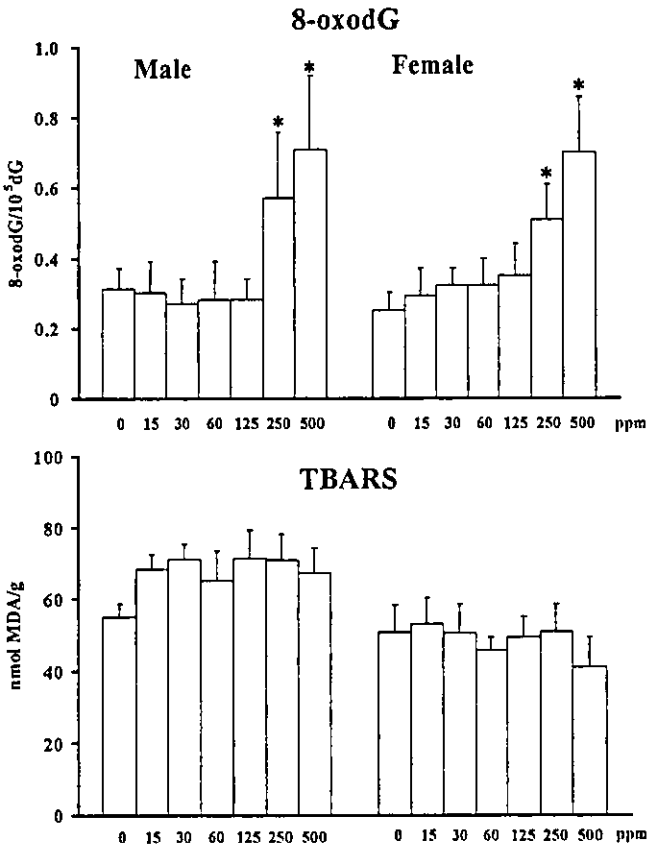

Fig. 2. 8-OxodG and TBARS levels in kidneys of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group (0 ppm).

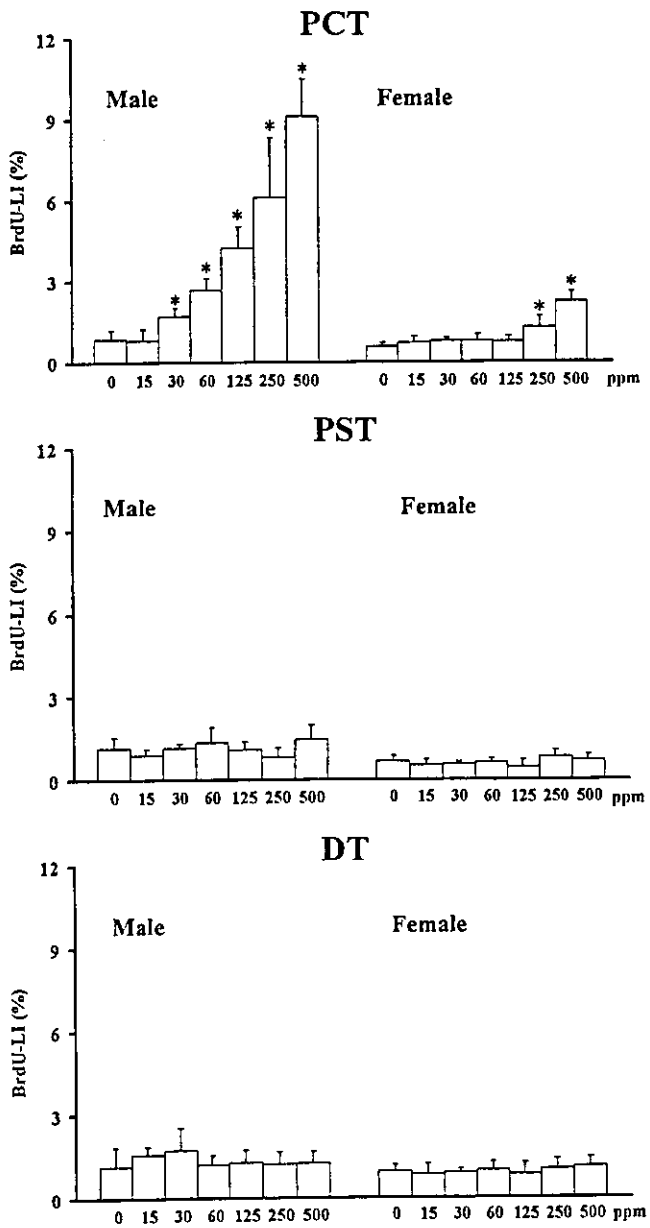

Fig. 3. BrdU-LIs for the proximal convoluted, straight and distal tubules (PCT, PST, DT) of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group (0 ppm).

which not only act as intermediates for free radical chain reactions, but also generate various reactive aldehydes, such as malondialdehyde and *trans*-4-hydroxy-2-nonenal, which directly form exocyclic DNA adducts.[22,32] A single exposure of male rats to $KBrO_3$ at high doses causes an increase of TBARS along with 8-oxodG formation,[27] which was also confirmed in the present study using female rats. However, exposure to carcinogenic doses in the drinking water failed to increase TBARS, in spite of the elevation of 8-oxodG levels. Another group has also reported that a single dose of $KBrO_3$ at a low dose did not elevate etheno-DNA adducts formation or TBARS levels in the kidneys of male rats.[21] In the light of the finding of no initiating activity of $KBrO_3$ with a single i.g. administration at 300 mg/kg,[33] our present data indicate that LPO might not be involved in the renal carcinogenesis due to this compound. Instead, histological findings in the present study suggest an involvement of LPO in the nephrotoxicity induced by $KBrO_3$. It has recently been reported that reduction of $KBrO_3$ by sulfhydryl compounds such as glutathione and cysteine yields bromine oxides and bromine radicals, which can effectively oxidize guanine.[34] A large amount of cysteine is supplied as a result of metabolism of glutathione by γ-glutamyltransferase on the proximal tubule brush borders,[35] where $KBrO_3$ reduction might give rise to bromine oxides. Since they are stable in comparison with radicals, they might move into the nuclei, where further reduction could generate bromine radicals in close proximity to nuclear DNA, leading to formation of 8-oxodG without any necessity for intervention of cellular LPO.

Kurokawa *et al.* earlier reported significantly elevated incidences of renal cell tumors in male and female rats given $KBrO_3$ at 250 and 500 ppm in the drinking water for 110 weeks.[2] A further dose-response study using only male rats showed 125 ppm to also be a carcinogenic dose.[36] However, a recent study by another group demonstrated that while $KBrO_3$

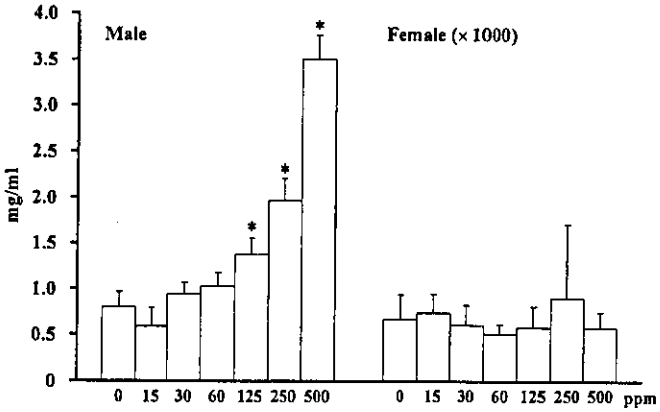

Fig. 4. α2u-Globulin levels in kidneys of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Note the values for females are μg/ml. Significantly different (* $P<0.01$) from the control group (0 ppm).

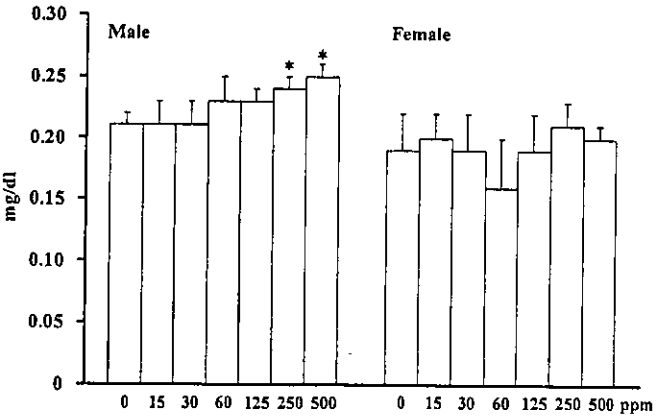

Fig. 5. Serum CRN levels in male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (*, ** $P<0.05$, 0.01) from the control group (0 ppm).

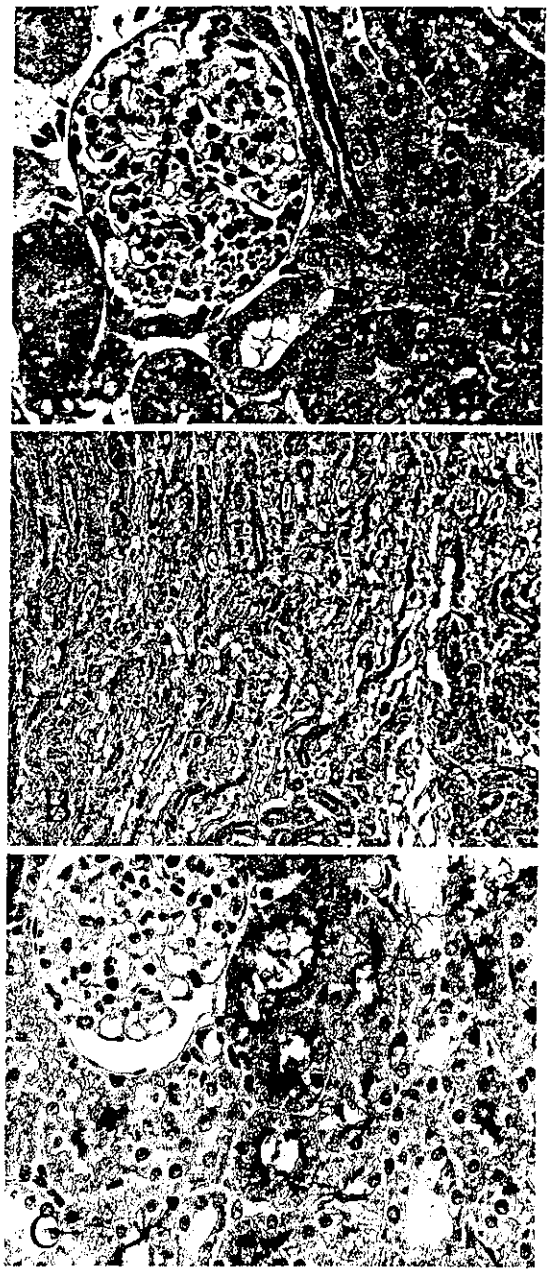

Fig. 6. (A) Renal cortex of a female rat treated with $KBrO_3$ at 80 mg/kg by single i.p. injection. Note hemorrhage and protein diapedesis in Bowman's capsule, accumulation of hyaline droplet-like material and basophilic alteration in proximal tubules. H&E staining at ×720 original magnification. (B) Renal medulla of a female rat treated with $KBrO_3$ at 80 mg/kg by single i.p. injection. Note extensive necrosis of collecting ducts. H&E staining at ×180 original magnification. (C) Renal cortex of a female rat treated with $KBrO_3$ at 500 ppm in drinking water for 4 weeks. BrdU-positive cells were seen in PCT (positive enzymatic reaction for γ-GT), but not in DT (negative enzymatic reaction for γ-GT). γ-GT-BrdU immunohistochemical staining at ×720 original magnification.

at 400 ppm in the drinking water was able to induce tumors in male rats with significant incidences, this was not the case with 200 ppm.[37] For the present, it seems equivocal whether 125 ppm has a carcinogenic potential. Accordingly, the present demonstration of increased 8-oxodG formation in kidney DNA of male and female rats given $KBrO_3$ at 250 and 500 ppm, but not at 125 ppm and below, seem to be in accordance with the carcinogenic data. In addition, the fact that $KBrO_3$-induced renal cell tumors originate from the proximal tubules[37] allows us to hypothesize that oxidative stress participates in the carcinogenesis. In a previous carcinogenicity study, the mean induction time for tumors in males was much shorter than in females,[2] but there was no sex difference with regard to doses inducing 8-oxodG formation in the present study. Therefore, variation in the tumor latency period might be explained by differential susceptibility to $KBrO_3$-induced cell proliferation, rather than oxidative stress.

In the two-stage rat renal carcinogenesis model using EHEN as an initiator, promoting activity of $KBrO_3$ was apparent in both sexes of rats.[24,25] In particular, in males, a dose of 30 ppm in the drinking water was sufficient for development of dysplastic foci from initiated cells. We also showed, in the present study, that $KBrO_3$ at the same dose was able to cause degeneration and increase of BrdU-LI in the PCT in the males. α2u-Globulin accumulation in the kidney of male rats exposed to $KBrO_3$ was also observed in a dose-dependent manner at 30 ppm and above, even though the increases at 30 and 60 ppm were not statistically significant. It has been established that this is associated with eventual cell death and subsequent cell proliferation.[38] Despite negative mutagenicity,[39,40] exposure to this kind of chemical can lead to renal cell tumors in male rats, which implies that α2u-globulin-mediated cell proliferation might be sufficient for tumor development.[41] Thus, it is highly probable that $KBrO_3$-induced cell proliferation in PCT and subsequent tumor-promoting activity observed in males might involve α2u-globulin accumulation. However, the finding that $KBrO_3$ exposure of female rats also increases BrdU-LI in PCT at doses of 250 and 500 ppm in spite of the absence of α2u-globulin indicates an involvement of some other mechanism. Considering that $KBrO_3$ might be reduced to form more reactive species at PCT,[34] the good correlation between the doses inducing 8-oxodG formation and elevation of BrdU-LI enables us to hypothesize that the cell proliferation observed in female rats might result from oxidative stress.[19,25] Since the histopathological findings and serum biochemical parameters indicate no obvious nephrotoxicity in female rats treated with $KBrO_3$ in the drinking water at any dose tested, oxidative stress might act via mitogenic stimulation.[42–44]

Judging from the female data, it appears that the cell proliferation observed in male rats at 125 ppm and below might be attributed to α2u-globulin accumulation and not to oxidative stress. In other words, the increase at 250 ppm and above in the males might reflect the combined effects of the two. For risk assessment of $KBrO_3$ in the human situation, it is essential to focus on oxidative stress and to ignore α2u-globulin-mediated effects.[45] The overall data allow us to hypothesize that more than 250 ppm of $KBrO_3$ in the drinking water is able to exert both initiating and promoting activities in the kidney of rats of both sexes by means of the generated oxidative stress. Long-term studies now appear warranted for confirmation.

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1. International Agency for Research on Cancer. Potassium bromate. *IARC Monogr Eval Carcinog Risks Hum* 1999; **73**: 481–96.
2. Kurokawa Y, Hayashi Y, Maekawa A, Takahashi M, Kokubo T. Induction of renal cell tumors in F-344 rats by oral administration of potassium bromate, a food additive. *Gann* 1982; **73**: 335–8.
3. The Ministry of Health and Welfare Japan. The Japanese Standards of Food Additives. 5th ed. The Ministry of Health and Welfare: Tokyo; 1986. p. 433.
4. Krasner SW, McGuire MJ, Jacagnelo JG, Patania NL, Reagan KM, Aieta EM. The occurrence of disinfection by-products in U.S. drinking water. *J Am Water Works Assoc* 1989; **81**: 41–3.
5. Jorgenson TA, Meierhenry EF, Rushbrook CJ, Bull RJ, Robinson M. Carcinogenicity of chloroform in drinking water to male Osborne-Mendel rats and female B6C3F1 mice. *Fundam Appl Toxicol* 1986; **5**: 760–9.
6. Carmichael NG, Winder C, Borges SH, Backhouse BL, Lewis PD. The health implications of water treatment with ozone. *Life Sci* 1982; **30**: 117–29.
7. Cavanagh JE, Weinberg HS, Gold A, Sangalah R, Marbury D, Glase WH, Collette TW, Richardson SD, Thruston AD. Ozonation byproducts: identification of bromohydrins from the ozonation of natural waters with enhanced bromide levels. *Environ Sci Technol* 1992; **26**: 1658–62.
8. Ishidate M, Sofuni T, Yoshikawa K, Hayashi M, Nohmi T, Sawada T, Matsuoka A. Primary mutagenicity screening of food additives currently used in Japan. *Food Chem Toxicol* 1984; **22**: 623–36.
9. Ishidate M, Yoshioka K. Chromosome aberration tests with Chinese hamster cells *in vitro* with and without metabolic activation: a comparative study on mutagens and carcinogens. *Arch Toxicol* 1980; **Suppl 4**: 41–4.
10. Hayashi M, Kishi M, Sofuni T, Ishidate M. Micronucleus tests with mice on 39 food additives and 8 miscellaneous chemical substances. *Chem Toxicol* 1988; **26**: 487–500.
11. Persons JL, Chipman JK. The role of glutathione in DNA damage by potassium bromate *in vitro*. *Mutagenesis* 2000; **15**: 311–6.
12. Ballmaier D, Epe B. Oxidative DNA damage induced by potassium bromate under cell-free conditions and in mammalian cells. *Carcinogensis* 1995; **16**: 335–42.
13. Kasai H, Nishimura S, Kurokawa Y, Hayashi Y. Oral administration of the renal carcinogen, potassium bromate, specifically produces 8-hydroxydeoxyguanosine in rat target organ DNA. *Carcinogenesis* 1987; **8**: 1959–61.
14. Cadenas S, Barja G. Resveratol, melatonin, vitamin E and PBN protect against renal oxidative DNA damage induced by the kidney carcinogen $KBrO_3$. *Free Radic Biol Med* 1999; **26**: 1531–7.
15. Arashidani K, Iwamoto-Tanaka N, Muraoka M, Kasai K. Genotoxicity of ribo- and deoxyribonucleosides of 8-hydroxyguanine, 5-hydroxycytosine, and 2-hydroxyadenine: induction of SCE in human lymphocytes and mutagenicity in *Salmonella typhimurium* TA100. *Mutat Res* 1998; **403**: 223–7.
16. Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G-T and A-C substitutions. *J Biol Chem* 1992; **267**: 166–72.
17. Le Page F, Margot A, Grollman AP, Sarasin A, Gentil A. Mutagenicity of a unique 8-oxoguanine in a human Ha-ras sequence in mammalian cells. *Carcinogenesis* 1995; **16**: 2779–84.
18. Nakae D, Umemura T, Kurokawa Y. Reactive oxygen and nitrogen oxide species-induced stress, a major intrinsic factor involved in carcinogenesis processes and a possible target for cancer prevention. *Asian Pacific J Cancer Prev* 2002; **3**: 313–8.
19. Umemura T, Takagi A, Sai K, Haesgawa R, Kurokawa Y. Oxidative DNA damage and cell proliferation in kidneys of male and female rats during 13-weeks exposure to potassium bromate ($KBrO_3$). *Arch Toxicol* 1998; **72**: 264–9.
20. Kurokawa Y, Takamura N, Matsuoka C, Imazawa T, Matsushima Y, Onodera H, Hayashi Y. Comparative studies on lipid peroxidation in the kidney of rats, mice, and hamsters and on the effects of cysteine, glutathione, and diethyl maleate treatment on mortality and nephrotoxicity after administration of potassium bromate. *J Am Coll Toxicol* 1987; **6**: 489–501.
21. Chipman JK, Davies JE, Parsons JL, Nair J, O'Neill G, Fawell JK. DNA oxidation by potassium bromate; a direct mechanism or linked to peroxidation? *Toxicology* 1998; **126**: 93–102.
22. Chung FL, Chen HJC, Nath RG. Lipid peroxidation as a potential endogenous source for the formation of exocyclic DNA adducts. *Carcinogenesis* 1996; **17**: 2105–11.
23. Moller P, Wallin H. Adduct formation, mutagenesis and nucleotide excision repair of DNA damage produced by reactive oxygen species and lipid peroxidation product. *Mutat Res* 1998; **410**: 271–90.
24. Kurokawa Y, Takahashi M, Kokubo T, Ohno Y, Hayashi Y. Enhancement by potassium bromate of renal tumorigenesis initiated by *N*-ethyl-*N*-hydroxyethylnitrosamine in F-344 rats. *Gann* 1983; **74**: 607–10.
25. Umemura T, Sai K, Takagi A, Hasegawa R, Kurokawa Y. A possible role for oxidative stress in potassium bromate ($KBrO_3$) carcinogenesis. *Carcinogenesis* 1995; **16**: 593–7.
26. Umemura T, Sai K, Takagi A, Hasegawa R, Kurokawa Y. A possible role for