— Regular Article —

Oxidation Products of *N*-nitrosodialkylamines Generated by Fenton's Reagent in the Presence of Copper Are Direct Acting Mutagens

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N-Nitrosodialkylamines, activated metabolically by cytochrome P450, possess mutagenic and carcinogenic activity. In this study, the hydroxyl radical, generated from Fenton's reagent, was used as an oxidant for the activation of the *N*-nitrosodialkylamines. Ethyl acetate extract from the reaction mixture which included Fe^{2+}-Cu^{2+}-H_2O_2 and *N*-nitrosodialkylamines; *N*-nitrosodimethylamine (NDM), *N*-nitrosodiethylamine (NDE), *N*-nitrosodipropylamine (NDP), *N*-nitrosodibutylamine (NDB), *N*-nitroso-*N*-methylpropylamine (NMP), *N*-nitroso-*N*-methylbutylamine (NMB), were assayed for their mutagenicity in *Salmonella typhimurium* (*S. typhimurium*) TA1535 and *Escherichia coli* (*E. coli*) WP2 *uvr*A. Although Fenton's reagent (Fe^{2+}-H_2O_2) alone did not activate NMB, the addition of the copper ion to the reaction with Fenton's reagent (Fe^{2+}-Cu^{2+}-H_2O_2) resulted in the production of mutagens. While the extracts of the reaction of NDM or NDE with Fe^{2+}-Cu^{2+}-H_2O_2 were not mutagenic, those of NMP, NDP, NMB, or NDB with Fe^{2+}-Cu^{2+}-H_2O_2 were mutagenic in both *S. typhimurium* TA1535 and *E. coli* WP2 *uvr*A. These results demonstrate that a direct-acting mutagen was formed from *N*-nitrosodialkylamines, with alkyl chains longer than propyl, by the oxidation in the Fe^{2+}-Cu^{2+}-H_2O_2 system.

Key words —— *N*-nitrosodialkylamine, metabolic activation, hydroxyl radical, Fenton's reagent, oxidation

INTRODUCTION

N-Nitrosamines are a class of mutagenic, teratogenic and carcinogenic chemicals that exist in the environment as contaminants in foods, tobacco or others.[1, 2] In addition to environmental exposure, human exposure to *N*-nitrosamines also occurs by nitrosation of amines in the body by reaction with nitrite in the presence of acid, or by reaction with products of nitric oxide generated during inflammation or infection.[3–10] *N*-Nitrosamines are alkylating agents which induce cancer in almost all experimental animals tested, and sometimes show target organ specificity based on their structure, *e.g.* *N*-nitrosodibutylamine (NDB) causes bladder cancer.[1, 11] Exposure to *N*-nitrosamines is therefore suspected to correlate with the induction of human cancers.[12–14]

The *N*-nitrosamines are classified structurally into two groups, *N*-nitrosamides and *N*-nitrosodialkylamines, both of which can induce DNA damage by alkylation.[1] *N*-Nitrosamides can spontaneously decompose and form the alkyldiazonium ion, while *N*-nitrosodialkylamines require metabolic activation through α-hydroxylation by cytochrome P450. Spontaneous decomposition of α-hydroxynitrosamines yields aldehydes and alkanediazohydroxides, followed by the generation of alkyldiazonium ions, which alkylate DNA bases. The O^6-alkylguanine formed caused GC-AT transition mutations early in the carcinogenic process.

Chemical oxidation systems have been used experimentally as an alternative metabolic system to elucidate the activation mechanisms of carcinogens.[15–17] Suzuki *et al.* used the Fenton and

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Udenfriend oxidation systems to determine the oxidation products of the urinary bladder carcinogenic NDB.[18] Therefore, we evaluated the mutagenic potencies of *N*-nitrosodialkylamines by the Fenton system in the presence or absence of copper ions, and investigated the effect of the metal ions and H_2O_2 on the mutagenicity of *N*-nitrosodialkylamines.

MATERIALS AND METHODS

Chemicals —— Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and hydrogen peroxide (H_2O_2) were purchased from Wako Chemical Co (Tokyo, Japan). Copper (II) acetate monohydrate [$Cu(OAc)_2 \cdot H_2O$] was obtained from Kanto Chemical Co. Ltd (Tokyo, Japan). *N*-Nitrosodimethylamine (NDM) was purchased from Tohshin Gousei (Tokyo, Japan). *N*-Nitrosodiethylamine (NDE) and *N*-nitrosodipropylamine (NDP) were kindly provided from Tokyo Biochemical Research Institute (Tokyo, Japan). *N*-Nitroso-*N*-methylpropylamine (NMP) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). NDB and *N*-nitroso-*N*-methylbutylamine (NMB) were synthesized from *N*,*N*-dibutylamine and *N*-methylbutylamine, respectively, using sodium nitrite in the presence of hydrochloric acid, then purified by distillation; NDB, b.p.105°C/8 mmHg; NMB, b.p. 87°C/18 mmHg.[19,20] Ames (University of California, Berkeley, CA, U.S.A.) kindly provided the *Salmonella typhimurium* (*S. typhimurium*) TA1535, and Iwahara (Hatano Research Institute, Food and Drug Safety Center, Hadano, Japan) kindly provided the *Escherichia coli* (*E. coli*) WP2 *uvr*A.

Reaction of *N*-nitrosodiakylamines with Fe^{2+}-Cu^{2+}-H_2O_2[18] —— To a solution of $FeSO_4 \cdot 7H_2O$ (0.28 g, 1 mmol) and $Cu(OAc)_2 \cdot H_2O$ (0.20 g, 1 mmol) in 1 M acetate buffer (pH 4.5, 18 ml), *N*-nitrosodialkylamine (1 mmol) was added, followed by the addition of 2% H_2O_2 (2 ml, 1 mmol) under an inert gas, and incubated for 2 hr at 37°C. The reaction mixture was extracted three times with ethyl acetate (10 ml) and the combined organic phase was washed with water (10 ml), dried over Na_2SO_4, filtered, and then evaporated to give a yellow oil.

Bacterial Mutation Assay[21] —— The bacterial mutation assay used was based on the Ames test.[21] The whole of the yellow oil obtained above was dissolved into dimethyl sulfoxide (DMSO, 20 ml) to give the solution containing the amount of original *N*-nitrosodialkylamine used in the reaction (1 mmol/20 ml), corresponding to 5.0 μmol/100 μl. This DMSO solution was diluted to the concentrations of 0.6, 1.3, and 2.5 μmol/100 μl. The each concentration of the DMSO solution was put into a test tube with a 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4), 0.1 ml of a culture of tester strain and 2 ml of top agar. The mixture was then poured onto a minimal-glucose agar plate. After incubation for 44 hr at 37°C, the colonies were counted. All plates were prepared in duplicate and the experiments were repeated at least twice. Data represent the means of duplicate determinations. The results are considered positive if the assay produced reproducible and dose-related increases in the number of revertants.[22]

Effect of Metal Ion and H_2O_2 on the Mutagenicity of *N*-nitrosodialkylamines —— The role of each component in the mutagenicity was investigated in the experiments of eliminating one component. *N*-Nitrosodialkylamine in the presence of the oxidation system were treated with neither $FeSO_4 \cdot 7H_2O$ nor $Cu(OAc)_2 \cdot H_2O$ nor H_2O_2. The optimum dose of each component was investigated in the experiments where the amount of Fe^{2+}, Cu^{2+} and H_2O_2, were varied. The extraction and the Ames assay were conducted the same as above.

RESULTS

Mutagenicity of *N*-nitrosodialkylamines in the Presence of Fe^{2+}-Cu^{2+}-H_2O_2

To investigate the structural requirement of *N*-nitrosodialkylamines for the mutagenicity, symmetric nitrosamines; NDM, NDE, NDP, NDB and asymmetric nitrosamines; NMP and NMB following the oxidation in the presence of Fe^{2+}-Cu^{2+}-H_2O_2, were tested in *S. typhimurium* TA1535 and *E. coli* WP2 *uvr*A (Fig. 1). In the symmetric nitrosamines, the oxidation products of NDM and

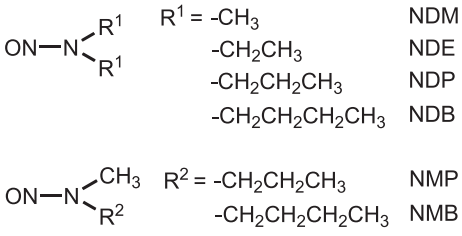

Fig. 1. Structures of *N*-nitrosodialkylamines Used in this Study

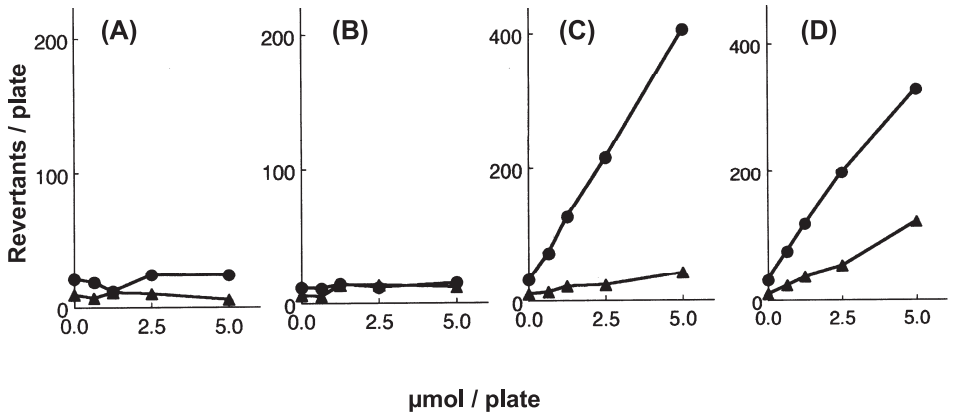

Fig. 2. Mutagenicity of the Oxidation Products Generated from the Reaction of Symmetric *N*-nitrosodialkylamines [NDM(A), NDE (B), NDP (C), NDB (D)] and Fe^{2+}-Cu^{2+}-H_2O_2 in *S. typhimurium* TA1535 (●) and *E. coli* WP2 *uvr*A (▲)

The oxidation products are the ethyl acetate extract derived from the reaction of *N*-nitrosodialkylamine (1 mmol) carried out under standard conditions: Fe^{2+} (1 mmol)-Cu^{2+} (1 mmol)-H_2O_2 (1 mmol) in 18 ml of 1 mM acetate buffer (pH 4.5). The abscissa showed the amount of *N*-nitrosodialkylamines in the original reaction solution in 100 μl DMSO of the extract.

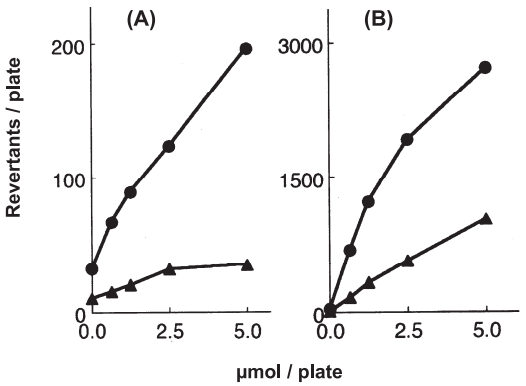

Fig. 3. Mutagenicity of the Oxidation Products Generated from the Reaction of Asymmetric *N*-nitrosodialkylamines [NMP (A), NMB (B)] and Fe^{2+}-Cu^{2+}-H_2O_2 in *S. typhimurium* TA1535 (●) and *E. coli* WP2 *uvr*A (▲)

The oxidation products are the ethyl acetate extract derived from the reaction of *N*-nitrosodialkylamine (1 mmol) carried out under standard conditions: Fe^{2+} (1 mmol)-Cu^{2+} (1 mmol)-H_2O_2 (1 mmol) in 18 ml of 1 mM acetate buffer (pH 4.5). The abscissa was similar to that in Fig. 1.

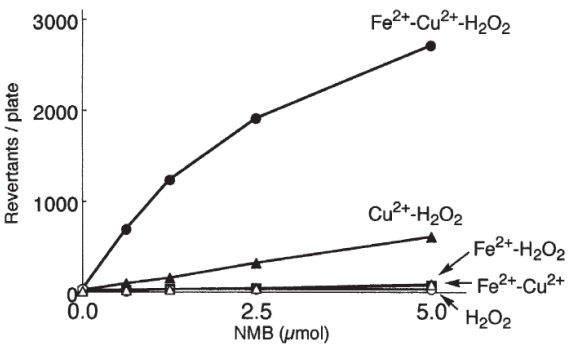

Fig. 4. Effects of Fe^{2+} and Cu^{2+} on Mutagenicity of the Oxidation Products of NMB in *S. typhimurium* TA1535

The oxidation products are the ethyl acetate extract derived from the reaction of NMB (1 mmol) in the presence or absence of H_2O_2 (1 mmol), Fe^{2+} (1 mmol) and Cu^{2+} (1 mmol) in 18 ml of 1 mM acetate buffer (pH 4.5). The abscissa was similar to that in Fig. 1.

NDE were not mutagenic, whereas the oxidation products of NDP and NDB were mutagenic, in both the *S. typhimurium* TA1535 and the *E. coli* WP2 *uvr*A strains (Fig. 2). The mutagenicity of asymmetric nitrosamines, NMP and NMB, the oxidation products was detected in *S. typhimurium* TA1535 and *E. coli* WP2 *uvr*A (Fig. 3).

The strength of the mutagenicity of the oxidation products was as follows; NMB > NDP > NDB > NMP. The result showed that an alkyl chain longer than propyl was necessary for the mutagenicity of nitrosamines activated by Fe^{2+}-Cu^{2+}-H_2O_2.

Effect of Metal Ion and H_2O_2 on the Mutagenicity of *N*-nitrosodialkylamines

The effect of the metal ions and H_2O_2 on the mutagenicity of the extracts from the reaction of NMB and Fe^{2+}-Cu^{2+}-H_2O_2 was investigated under the conditions with a possible combination of the components present during the oxidation reaction (Fig. 4). Cu^{2+}-H_2O_2 weakly activated NMB, however, Fe^{2+}-H_2O_2, Cu^{2+}-Fe^{2+}, or H_2O_2 alone did not activate NMB (Fig. 4). The coexistence of both metal ions and H_2O_2 greatly enhanced the mutagenicity of the extracts from the reaction with NMB. Thus, Fe^{2+} and Cu^{2+} have a synergistic effect on the formation of the mutagen.

DISCUSSION

N-Nitrosodialkylamines are pro-carcinogens that require metabolic activation by cytochrome P450 enzymes to exert their mutagenicity and carcinogenicity. The α-hydroxylation of carbon atoms adjacent to the *N*-nitroso group is believed to result in the metabolic activation of *N*-nitrosodialkylamines, followed by the formation of alkanediazohydroxide, which alkylates DNA.

Oxidation of several dialkynitrosamines, including NDB, by chemical model systems was first investigated by Preussmann, who observed no reaction with the Fenton system, although several products were formed from NDB in the Udenfriend system, as determined by TLC.[15] Manson *et al.* compared the oxidation of *N*-nitrosomorpholine by rat liver microsomes and the Fenton system, and observed its conversion to *N*-nitroso-2-hydroxymorpholine by both systems.[17] Suzuki *et al.* reported the formation of *N*-butyl-*N*-(3-oxobutyl)nitrosamine as the major products of NDB oxidation in the Fenton system supplemented with Cu^{2+}.[18] In the current study, we investigated the activation of the *N*-nitrosodialkylamines in the presence of Fe^{2+}-Cu^{2+}-H_2O_2.

The ethyl acetate extract of the reaction mixture of NDP, NMP, NDB and NMB with Fe^{2+}-Cu^{2+}-H_2O_2 was mutagenic in *S. typhimurium* TA1535 and *E. coli* WP2 *uvr*A. The results suggested that the *N*-nitrosodialkylamines containing an alkyl chain longer than propyl was mutagenic following the oxidation in the presence of Fe^{2+}-Cu^{2+}-H_2O_2. Both *S. typhimurium* TA1535 and *E. coli* WP2 *uvr*A are known to detect base substitution mutations;[23] therefore, the direct-acting mutagen has been shown to cause a base substitution, similar as the ultimate active species α-hydroxynitrosamine. All mutagens showed no mutagenicity in the absence of the activation in *S. typhimurium* TA1535 and *E. coli* WP2 *uvr*A. The oxidation products derived from NMB were observed by TLC and HPLC. The data indicated that the nitrosamines were activated by Fe^{2+}-Cu^{2+}-H_2O_2. The oxidation products appeared to be more potent mutagens in *Salmonella*, as compared to *E. coli*. NDM, NDE, NDP and NDB were mutagenic in the presence of S9 mix; however, the activity of NDE, NDP and NDB was found to be stronger in *E. coli* than in the *Salmonella*.[23] The relative strength of the mutagenicity in the two strains was different in the presence of S9 mix and of Fe^{2+}-Cu^{2+}-H_2O_2. The direct-acting mutagen formed in the presence of Fe^{2+}-Cu^{2+}-H_2O_2 was not the α-hydroxynitrosamine, which is highly unstable in aqueous solution.[24] It is interesting that the mutagenicity of the oxidation products of NDB generated in the presence of Fe^{2+}-Cu^{2+}-H_2O_2 produced the same alkylating damage observed with the α-hydroxynitrosamines.

The direct-acting mutagen was formed from NMB in the presence of Fe^{2+}-Cu^{2+}-H_2O_2, but was not formed in the system lacking the copper ion. Although the pattern of main products in the modified Fenton systems was essentially the same,[18] the mutagenic products were formed only in the presence of Fe^{2+}, Cu^{2+} plus H_2O_2. Since Walling *et al.* reported that the oxidation with Fenton system supplemented by copper ion is a different process than the system without the copper ion, the NDB oxidation products are most likely different from those formed in the presence of copper.[25, 26] In the current study, Cu^{2+} appeared to have played a key role in the formation of the direct-acting mutagen. The structure of the direct-acting mutagen is under investigation.

As we detected the direct-acting mutagen in this study, the chemical model system has an advantage to identify the unknown mutagen in the reaction mixture and opens a possibility to find a new activation pathway.

In conclusion, the oxidation products of the reaction of *N*-nitrosodialkylamines in the presence of Fe^{2+}-Cu^{2+}-H_2O_2, which have an alkyl chain longer than propyl, were mutagenic in both *S. typhimurium* TA1535 and *E. coli* WP2 *uvr*A. The mutagenic products are not α-hydroxynitrosamines, as those molecules are highly unstable in aqueous solutions. Thus, there is a possibility that the direct-acting mutagen formed via the oxidation of *N*-nitrosodialkylamines represents a novel product in the oxidative activation.

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