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Mutagenicity of isomeric alkanediazotates, precursors for ultimate alkylating species of carcinogenic *N*-nitroso compounds [1]

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Abstract

Alkanediazohydroxides are common key intermediates in carcinogenesis and mutagenesis of *N*-nitroso compounds, which are widely found in human environment. Mutagenicity of (*E*)- and (*Z*)-potassium alkanediazotates, as precursors of corresponding alkanediazohydroxides were evaluated to investigate the effect of geometric isomerism and also the effect of alkyl groups on their biological activity. Mutagenicity of *N*-nitroso-*N*-alkylureas which spontaneously produce alkanediazohydroxides after non-enzymatic hydrolysis were also tested in comparison to that of the corresponding diazotates and other activated chemical species of *N*-nitrosamines. When the mutagenicity was assayed in three microbial strains, *Salmonella typhimurium* TA1535, and *Escherichia coli* WP2 and WP2 *uvrA*, the order of mutagenic potency of the compounds with the same alkyl group was as follows; (*E*)-diazotates > (*Z*)-diazotates > nitrosoureas. The effect of alkyl groups on the mutagenic potency was different in *Salmonella* strain and in *E. coli* strains, and this result could be explained by the efficiency of O^6-alkylguanine-DNA alkyltransferase. In each bacterial strain, this effect of alkyl groups was similar in mutagenicity induced by (*E*)- and (*Z*)-diazotates, *N*-nitroso-*N*-alkylureas and other activated *N*-nitrosodialkylamines such as α-hydroxy nitrosamines. The geometrical isomerism affected the mutagenicity of (*E*)- and (*Z*)-potassium alkanediazotates, and the result suggested that alkanediazohydroxides react through diazonium ions in a cage rather than through free alkyldiazonium ions which have no geometrical isomerism. Our results confirmed that (*E*)-potassium alkanediazotates, (*Z*)-potassium alkanediazotates and *N*-nitroso-*N*-alkylureas all decomposed through diazohydroxides, and that alkanediazohydroxides are the active alkylating species of *N*-nitroso compounds, and also that the geometrical isomerism is important for carcinogenic *N*-nitroso compounds to show their biological activity. © 1998 Elsevier Science B.V.

Keywords: Alkanediazotate; Ultimate alkylating species; *N*-Nitroso compound; Mutagenicity; Geometrical isomerism

1. Introduction

N-Nitroso compounds are strong carcinogens for animals, and are found in our environment such as foods, drugs, or tobacco smoke [2]. They are also formed in vivo under acidic conditions in stomach by the reaction of amines in foods and drugs, with nitrite arising from nitrate in human saliva [3,4], or under neutral conditions by the action of activated macrophages on amines [5,6]. Thus *N*-nitroso compounds are considered to be a possible cause of human cancer [7].

N-Nitroso compounds are classified into two

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groups; *N*-nitrosamines and *N*-nitrosamides. *N*-Nitrosamines need metabolic activation to show any biological activity [8]. *N*-Nitrosodialkylamines, one major group of *N*-nitrosamines, are metabolically hydroxylated by cytochrome P450 2E1 at α carbon, forming active metabolites, α-hydroxy nitrosamines. The α-hydroxy nitrosamines decompose to alkanediazohydroxides, and then to alkyldiazonium ions, which alkylate DNA or other biological molecules (Fig. 1) [8,9]. Since α-hydroxy nitrosamines are quite unstable in aqueous solution, their acetyl esters, α-acetoxy nitrosamines, have been used as precursors for α-hydroxy nitrosamines in research for chemical and biological activities of *N*-nitrosamines [10]. α-Hydroperoxy nitrosamines were synthesized by nucleophilic substitution of the corresponding α-acetoxy nitrosamines with hydrogen peroxide in acetic acid [11], and also by oxygenation of dialkylnitrosamines in the presence of a strong base [12]. The α-hydroperoxy nitrosamines also showed strong mutagenicity in bacterial strains [13]. The α-hydroxy nitrosamines were isolated by deoxygenation of α-hydroperoxy nitrosamines [13–15], and their chemistry and mutagenicity have been elucidated [15].

Alkanediazohydroxides have been thought to be important intermediates in alkylation of carcinogenic *N*-nitroso compounds. They have geometrical isomers, *E* and *Z*, due to a double bond character of N=N bond [16]. Although diazohydroxides are quite unstable to isolate, potassium alkanediazotates of each isomer can be synthesized [17,18]. The synthesis of alkanediazotates was reported in 1910 by Thiele [17], then studies about their structure and chemistry were reported [16,19,20], Recently Fishbein and co-workers reported some studies on decomposition of simple alkanediazotates in aqueous solution [21–23]. Since the alkanediazohydroxides may be common key intermediates in carcinogenesis or mutagenesis of *N*-nitroso compounds, it is important to investigate the relation between their geometry and biological responses. Because alkanediazotates are easily converted to the corresponding alkanediazohydroxides in aqueous solution, they are useful precursors for investigation of behavior of alkanediazohydroxides [24]. However, few studies about biological activity related to its chemical reactivity were reported so far, except the mutagenicity of (*Z*)-alkanediazotates reported in 1983 by Hecker et al. [24].

This paper describes the mutagenicity of the two geometrical isomers of diazotates, (*E*)- and (*Z*)-potassium alkanediazotates, concerning the effect of geometry and their alkyl groups on biological activity. The effect of alkyl groups was compared with

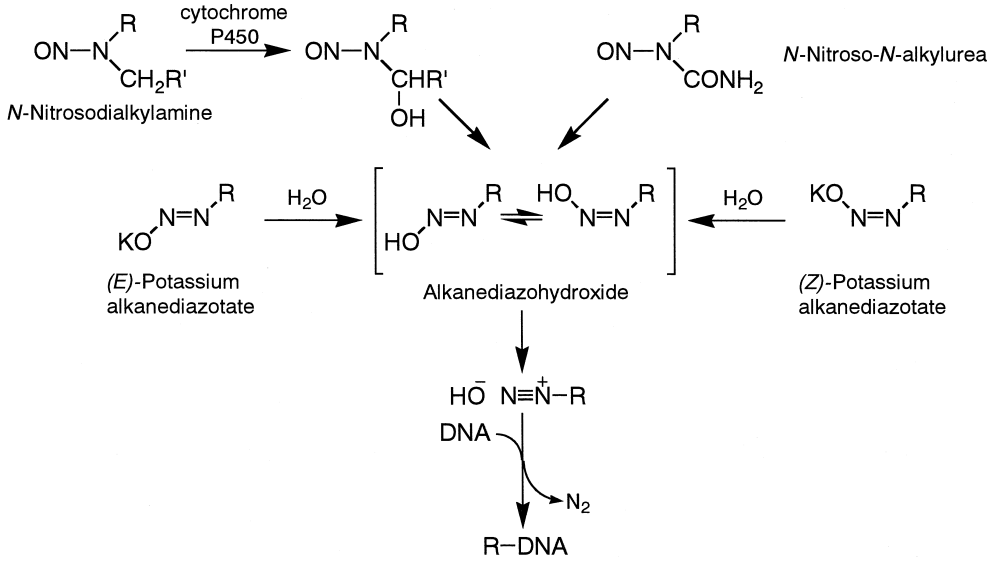

Fig. 1. Activation of carcinogenic *N*-nitroso compounds.

that of the corresponding *N*-nitroso-*N*-alkylureas or activated *N*-nitrosodialkylamines to confirm the role of alkanediazohydroxides in mutagenesis or carcinogenesis of *N*-nitroso compounds.

2. Materials and methods

2.1. Chemicals

Most reagents used were purchased from Wako Pure Chemical (Osaka, Japan) as the purest grade available. Methylhydrazine was purchased from Aldrich Chemical (Milwaukee, WI, USA). Ethylhydrazine oxalate, propylhydrazine oxalate and butylhydrazine oxalate were purchased from Fluka Chemie (Buchs, Switzerland). *N*-Nitroso-*N*-methyl-*p*-toluenesulfonamide and *N*-butyl-*p*-toluenesulfonamide were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and *N*-ethyl-*p*-toluenesulfonamide was purchased from Eastman Kodak (Rochester, NY, USA). *N*-Nitroso-*N*-alkylureas having methyl, ethyl, propyl and butyl groups were kindly provided by Toshin Chemical Industry (Tokyo, Japan). Ethanol and dimethyl sulfoxide were freshly distilled from calcium hydride before use. Bacto agar and nutrient broth were purchased from Difco Laboratories (Detroit, MI, USA), and sodium ammonium hydrogenphosphate was purchased from Merck (Rahway, NJ, USA).

2.2. (E)-Potassium alkanediazotates

(*E*)-Potassium methanediazotate (CAS 98114-62-6) was synthesized by the methods reported [17]. Methylhydrazine was dissolved in anhydrous ether, and was added by freshly prepared etherial solution of potassium ethoxide, then was added by alcoholic solution of ethyl nitrite. The mixture was stirred for 2 h at 0°C, and the resulting precipitate was filtered under nitrogen atmosphere, and washed with anhydrous ether. White solid obtained was used without further purification. Purity was calculated by integration of ^{1}H-NMR data adding tri-*t*-butylbenzene as an internal standard. Purity: 70.3%. Yield: 15.6%. ^{1}H-NMR (DMSO-d_6) (ppm): 3.10 (3H, singlet) [21].

Other (*E*)-diazotates having ethyl, propyl and butyl groups were synthesized by the methods described above except using *N*-nitroso-*N*-alkylhydrazines as starting materials. *N*-Nitroso-*N*-alkylhydrazines (alkyl = ethyl, propyl and butyl) were synthesized by nitrosation of the corresponding alkylhydrazine in acetic acid [17,25,26]. *N*-Nitroso-*N*-alkylhydrazine prepared was dissolved in anhydrous ether, and was added by etherial solution of potassium ethoxide and alcoholic solution of ethyl nitrite. The mixture was stirred for 2 h at 0°C, and the resulting precipitates were filtered, and washed with anhydrous ether. White solid obtained was used without further purification. Purity was calculated by integration of ^{1}H-NMR data adding tri-*t*-butylbenzene as an internal standard. (*E*)-Potassium ethanediazotate (CAS 92078-92-7). Purity: 32.8%. Yield: 23.2%. ^{1}H-NMR (DMSO-d_6) (ppm): 0.98 (3H, triplet), 3.38 (2H, quartet). (*E*)-Potassium propanediazotate (CAS 98114-63-7). Purity: 76.2%. Yield: 21.4%. ^{1}H-NMR (DMSO-d_6) (ppm): 0.84 (3H, triplet), 1.42 (2H, sextet), 3.34 (2H, triplet). (*E*)-Potassium butanediazotate (CAS 98114-64-8). Purity: 55.9%. Yield: 32.2%. ^{1}H-NMR (DMSO-d_6) (ppm): 0.87 (3H, triplet), 1.28 (2H, sextet), 1.40 (2H, quintet), 3.38 (2H, triplet) [23].

2.3. (Z)-Potassium alkanediazotates

(*Z*)-Potassium methanediazotate (CAS 3058-37-5) was synthesized by the methods reported [18]. *N*-Nitroso-*N*-alkyl-*p*-toluenesulfonamide having ethyl, propyl and butyl groups was prepared from the corresponding *N*-alkyl-*p*-toluenesulfonamide (ethyl and butyl) by nitrosation, or nitrosation after alkylation of *p*-toluenesulfonamide (propyl) [27]. *N*-Nitroso-*N*-alkyl-*p*-toluenesulfonamide was dissolved in anhydrous ether, and was added by freshly prepared etherial solution of potassium ethoxide. The mixture was stirred for 2 h at 0°, and the resulting precipitate was filtered, and washed with anhydrous ether. White solid obtained was used without further purification. Purity was calculated by integration of ^{1}H-NMR data adding tri-*t*-butylbenzene as an internal standard. (*Z*)-Potassium methanediazotate. Purity: 17.8%. Yield: 17.4%. ^{1}H-NMR (DMSO-d_6) (ppm): 2.63 (3H, singlet) [18]. (*Z*)-Potassium ethanediazotate (CAS 94610-15-8). Purity: 31.7%. Yield: 6.7%. ^{1}H-NMR (DMSO-d_6) (ppm): 1.02 (3H, triplet),

3.07 (2H, quartet). (*Z*)-Potassium propanediazotate (CAS 98114-60-4). Purity: 38.1%. Yield: 13.8%. ^{1}H-NMR (DMSO-d_6) (ppm): 0.86 (3H, triplet), 1.47 (2H, sextet), 2.99 (2H, triplet) [24]. (*Z*)-Potassium butanediazotate (CAS 98114-61-5). Purity: 46.5%. Yield: 31.4%. ^{1}H-NMR (DMSO-d_6) (ppm): 0.86 (3H, triplet), 1.47 (2H, sextet), 1.62 (2H, quintet), 3.05 (2H, triplet).

2.4. Bacterial strains

A culture of *Salmonella typhimurium* TA1535 was kindly provided by Dr. B.N. Ames, University of California, Berkeley, USA. *Escherichia coli* WP2 and WP2 *uvrA* were kindly donated by Dr. S. Iwahara, Food and Drug Safety Center, Hadano, Japan.

2.5. Mutation assay

The medium used for overnight culture of bacterial strains was a nutrient broth medium (NB) containing 6 g of nutrient broth and 5 g of NaCl per liter. The minimal media used for mutation assays were as follows: *S. typhimurium* strain, the medium reported by Maron and Ames [28]; *E. coli* strains, modified Vogel–Bonner E medium supplemented with 20 ml of NB per liter and 0.4% glucose. The agar plates contained 30 ml of the minimal medium containing 1.5% Bacto agar.

The bacteria were grown in 5 ml of NB for 15.5 h at 37°C. Test compounds were diluted in dimethyl sulfoxide (DMSO). To a tube containing 0.5 ml of 0.1 M sodium phosphate buffer (pH7.4) and 0.1 ml of a culture of bacterial tester strain, 0.1 ml of sample solution was added, then the mixture was shaken immediately, and was mixed with 2 ml of top agar (0.6% agar in 0.6% NaCl) at 45°C and was poured on a minimal agar plate. After incubation at 37°C for 2 days, the colonies on the plate were counted manually, and the number of spontaneous revertant colonies was subtracted from those of the induced revertants. Concentration of the test compounds were expressed as μmol/plate. All data reported are representative of at least three experiments using duplicate plates per each dose level.

3. Results

The mutagenicity of (*E*)- and (*Z*)-potassium alkanediazotates and *N*-nitroso-*N*-alkylureas was assayed in *S. typhimurium* TA1535, and *E. coli* WP2 and

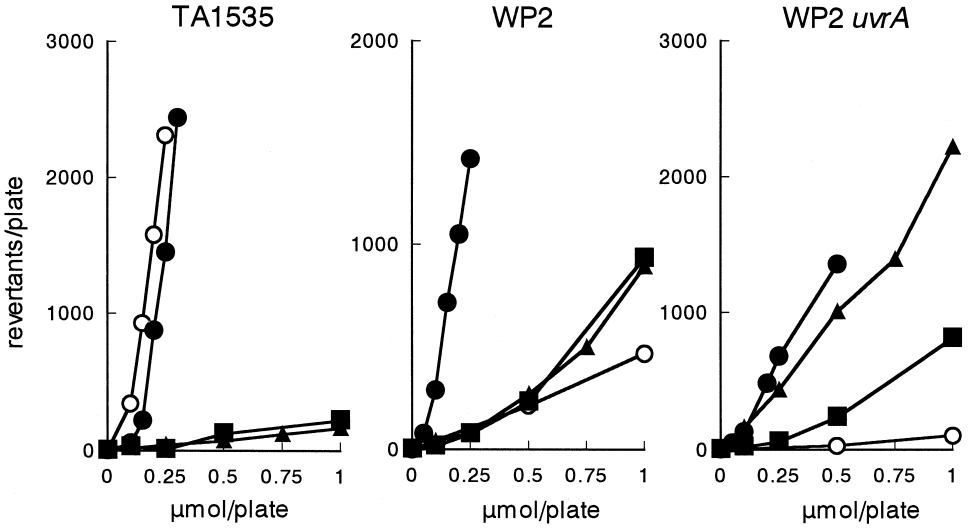

Fig. 2. Mutagenicity of (*E*)-potassium alkanediazotates in three different microbial strains: *S. typhimurium* TA1535, *E. coli* WP2 and WP2 *uvrA*. ○, Methanediazotate; ●, ethanediazotate; ■, propanediazotate; ▲, butanediazotate.

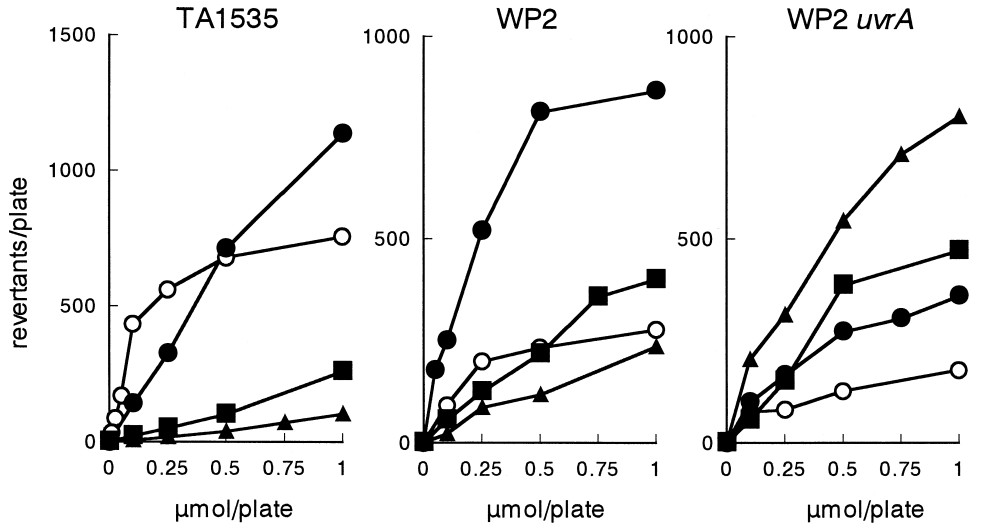

Fig. 3. Mutagenicity of (*Z*)-potassium alkanediazotates in three different microbial strains: *S. typhimurium* TA1535, *E. coli* WP2 and WP2 *uvrA*. ○, Methanediazotate; ●, ethanediazotate; ■, propanediazotate; ▲, butanediazotate.

WP2 *uvrA*. All compounds were mutagenic, and the mutagenic potency was linearly related to the concentration of chemicals (Figs. 2–4). A specific mutagenicity per μmol of chemicals was defined by the slope of the linear part in the dose–mutagenicity relation and was calculated by the least squares method. Fig. 5 shows the specific mutagenicity for three series of chemicals in three different microbial strains. In all strains, (*E*)-diazotates were most mutagenic, and the activity was ten times stronger than that of the corresponding (*Z*)-diazotates. *N*-Nitroso-*N*-alkylureas were less mutagenic than diazotates. In

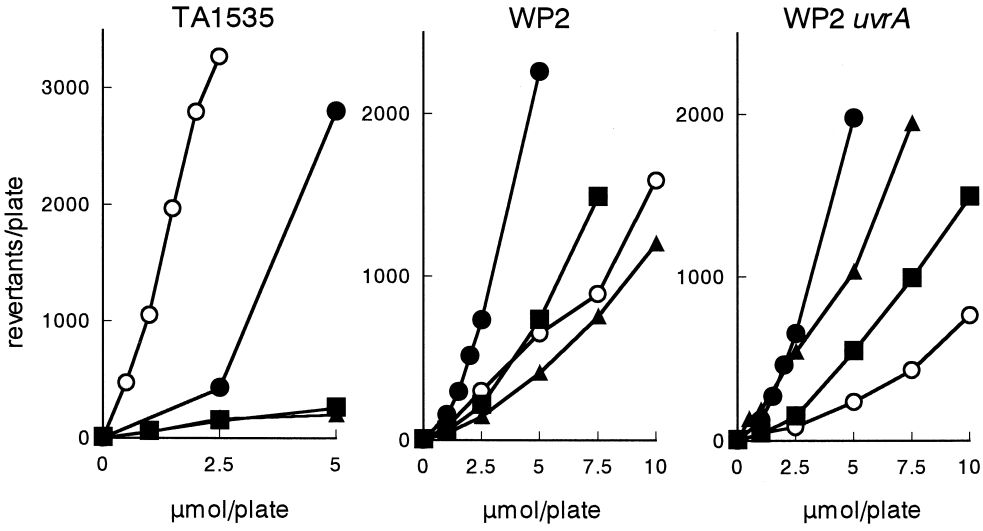

Fig. 4. Mutagenicity of *N*-nitroso-*N*-alkylureas in three different microbial strains: *S. typhimurium* TA1535, *E. coli* WP2 and WP2 *uvrA*. ○, Nitrosomethylurea; ●, nitrosoethylurea; ■, nitrosopropylurea; ▲, nitrosobutylurea.

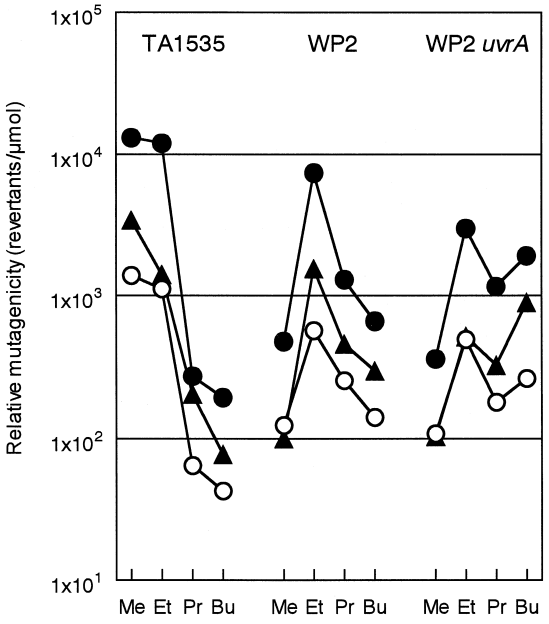

Fig. 5. Relative mutagenicity of (*E*)-potassium alkanediazotates (●), (*Z*)-potassium alkanediazotates (▲) and *N*-nitroso-*N*-alkylureas (○) in *S. typhimurium* TA1535, *E. coli* WP2 and WP2 *uvrA* strains. Revertants per μmol were calculated by the least-squares method from the linear part in the dose–mutagenicity relationship.

S. typhimurium TA1535 strain, increase of alkyl chain length decreased the mutagenic potency. In *E. coli* strains, ethyl homologues were more mutagenic than methyl homologues, while in WP2 *uvrA* strain, butyl homologues were more mutagenic than propyl homologues (Fig. 5).

4. Discussion

Hecker et al. already reported the mutagenicity of (*Z*)-potassium alkanediazotates having methyl, ethyl and propyl groups as precursors of activated nitrosamines [24]. In the present study, we systematically and quantitatively evaluated the mutagenicity of both geometrical isomers, (*E*)- and (*Z*)-potassium alkanediazotates, having methyl, ethyl, propyl and butyl groups with the purity determined by NMR spectroscopy, together with *N*-nitroso-*N*-alkylureas which decompose to alkanediazohydroxides as common intermediates in the metabolism of *N*-nitrosodialkylamines. The strains used by Hecker et al. were *S. typhimurium* TA1535 and TA100, which can detect base-pair change mutagenicity [28]. Although strain TA100 are commonly used in many investigations, we selected strain TA1535 to exclude the effect of SOS response due to R-factor plasmid pKM101. The two *E. coli* strains, WP2 and WP2 *uvrA*, can also detect base-pair change mutagenicity [29], and they were used together with TA1535 strain for the comparison to the mutagenicity of the series of activated *N*-nitrosamines already reported [10,13,15]. Both *S. typhimurium* TA1535 and *E. coli* WP2 *uvrA* are deficient in excision repair system [28,29].

(*E*)- and (*Z*)-potassium alkanediazotates and *N*-nitroso-*N*-alkylureas were all mutagenic in three microbial strains, and the difference in alkyl group affected their mutagenicity. The activity of (*Z*)-ethanediazotate was higher than that of reported result [24], since our determination of purity of diazotates by using internal standard allowed the accurate calculation of molar concentration for mutation assay. When the alkyl group was changed, the mutagenic activity was decreased with an increase of alkyl chain length in *S. typhimurium* TA1535. The effect of changing alkyl groups on relative mutagenicity described above was similar in two series of diazotates and nitrosoureas, and also in other activated *N*-nitrosodialkylamines, such as α-hydroxy nitrosamines [13], α-acetoxy nitrosamines [10] and α-hydroperoxy nitrosamines [13]. The effects were explained by reactivity and selectivity of alkyl groups by nucleophiles. The rate of alkylation of deoxyguanosine by α-hydroxy nitrosamine was decreased by the following order: methyl > ethyl > propyl > butyl [13]. Furthermore, in the hard and soft acids and bases (HSAB) principle [30], alkyl cations with more carbon branches were harder acids [31,32], and the N^7-position of guanine was a harder base, while the O^6-position of guanine was a softer base [19]. Thus, a softer methyl group could more effectively alkylate the O^6-position of guanine to make methanediazotate the most mutagenic.

While, in *E. coli*, ethyl homologues were more mutagenic than methyl homologues (Fig. 5). The difference between the response in *Salmonella* strain and that in *E. coli* strains was explained by the efficiency of O^6-alkylguanine-DNA alkyltransferase.

The enzyme activity for the repair in *E. coli* was inducible in contrast to that in *S. typhimurium* [33]. Therefore, the repair enzyme of *E. coli* was effectively induced in the conditions of the present study, so the methyl homologue was less mutagenic than ethyl homologue.

N-Nitrosamides, such as *N*-nitroso-*N*-methylurea or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, are chemically more reactive than *N*-nitrosamines, and do not require any metabolic activation. They decompose spontaneously through alkanediazohydroxides under physiological conditions, and subsequently alkylate nucleophiles in vivo [34] (Fig. 1). There are several reports about mutagenicity of *N*-nitroso-*N*-methylurea or simple *N*-nitrosoureas having larger alkyl groups such as ethyl, propyl and butyl groups [35–37]. Our results that *N*-nitroso-*N*-methylurea was most mutagenic in *S. typhimurium* TA1535, and that *N*-nitroso-*N*-ethylurea was most mutagenic in *E. coli* strains, were identical with the reported results. In this paper, the mutagenicity of *N*-nitroso-*N*-alkylureas was examined under the same conditions for comparison with those of diazotates, and the effect of alkyl groups on mutagenicity was similar to that of (*E*)- and (*Z*)-diazotates. These results supported that *N*-nitrosoureas decomposed to the corresponding alkanediazohydroxides, and then showed their alkylating activity.

(*E*)-Potassium alkanediazotates were more mutagenic than (*Z*)-diazotates. The result suggested that geometrical isomerism affected biological activity such as mutagenicity or carcinogenicity. The reactivity toward nucleophiles is higher in (*Z*)-diazotates than in (*E*)-diazotates, because (*Z*)-diazotates with softer carbon react with the O^6-position of guanine more easily than (*E*)-diazotates with harder carbon estimated by HSAB principle and ab initio calculation [19]. In spite of the greater reactivity of (*Z*)-diazotates, most (*Z*)-diazotates have shorter lifetimes than (*E*)-diazotates in aqueous media used as solvent in mutation assay, because the anti-periplanar arrangement of lone pair of electrons on nitrogen of (*Z*)-diazotate assists easy elimination of the hydroxyl group from (*Z*)-diazohydroxide, conjugated acid of diazotate [23]. Thus, the weaker mutagenicity of (*Z*)-diazotates could be explained by their instability.

Besides alkanediazohydroxides or alkanediazotates, non-symmetrical *N*-nitrosodialkylamines (or their α-hydroxy derivatives) also have geometrical isomers due to N–N=O bond. The actual structure of N–N=O is a resonance hybrid among two resonance structures with a single N–N bond and a double N=N bond, then the N–N bond is realized as a partial double bond. It is unknown whether the geometry of *N*-nitrosamines is retained when they convert to alkanediazohydroxides, however, our results suggested that the geometry of the reactive intermediate possibly affected the biological activity of carcinogenic nitrosamines. In α-acetoxy nitrosamines, the ratio of *Z*-isomer to *E*-isomer increased by substitution of bulky alkyl group [38], and the higher the ratio of *Z*-isomer in α-acetoxy nitrosamine, the faster was their rate of decomposition [39]. Farrelly et al. [40] reported that the difference in the rate of metabolism of (*E*)- and (*Z*)-isomers for *N*-nitroso-*N*-methyl-2-oxopropylamine, and *N*-nitroso-*N*-methyl-*N*-*n*-pentylamine behaved similarly [41]. (*E*)- and (*Z*)-diazotates cannot easily isomerize to each other [19], so it is of interest to determine whether *N*-nitroso compounds decompose to alkanediazohydroxides retaining or inverting their geometric form. Since few studies about the relation of geometrical isomerism of *N*-nitroso compounds and their related biological properties were reported, the present results revealed that geometrical isomerism of alkanediazohydroxides greatly affected on their biological activities.

The results that mutagenicities of (*E*)- and (*Z*)-diazotates were different suggested that alkanediazohydroxides may not react through free alkyldiazonium ions which have no geometrical isomerism but through diazonium ions in a cage, and a possible reactive form in protic solvent is an ion triplet proposed by Moss [16], which retains the nature of steric structure of (*E*)- or (*Z*)-isomer.

Although the mutagenic potency of a series of *N*-nitrosamines, *N*-nitroso-*N*-alkylureas, or alkanediazotates were different quantitatively, the effect of alkyl groups on their mutagenicity in three microbial strains was identical. Present results using alkanediazotates as precursors for alkanediazohydroxides further supported that carcinogenic *N*-nitroso compounds decompose through alkanediazohydroxides, and that alkanediazohydroxides are the common active alkylating species of *N*-nitroso compounds. In addition, the present result that geometrical iso-

merism of alkanediazotates affected on bacterial mutagenicity suggested that alkanediazohydroxide or alkyldiazonium ion in a cage [16] is a possible ultimate alkylating species rather than free alkyldiazonium ion, and that the geometry of alkanediazohydroxides might determine biological behaviors of their parent *N*-nitroso compounds.

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