A Carbon-Centered Radical as a Reaction Species in the DNA Strand-Breakage by D-Glucosamine⁵⁾

Tadatoshiki YAMAGUCHI,^{*,a} Nobuhiro KASHIGE,^b Shigenobu MATSUMOTO,^c Kazue SATOH,^d Masahide YASUDA,^e and Kenji WATANABE^b

Department of Hygiene, Miyazaki Medical College,^a Kiyotake-cho, Miyazaki 889–16, Faculty of Pharmaceutical Sciences, Fukuoka University,^b Fukuoka 814–80, Department of Biochemistry and Isotope, Tokyo Metropolitan Institute of Gerontology,^c Itabashi-ku, Tokyo 173, Analysis Center, School of Pharmaceutical Sciences, Showa University,^d Shinagawa-ku, Tokyo 142, and Cooperative Research Center, Miyazaki University,^e Miyazaki 889–21, Japan.

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Electron spin resonance (ESR) studies using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and sodium 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS) as a spin-trapping agent revealed the formation of both hydroxyl and carbon-centered radical-derived spin adducts in Cu^{2+} -containing 50 mM Tris–HCl buffered solutions (pH 7.1) of D-glucosamine, D-mannosamine, and D-galactosamine, which were previously shown to have the ability to break the single-strand of plasmid pBR322 DNA in a nucleotide sequence-specific manner.

HCl-free D-glucosamine has higher DNA breaking activity, and this activity is promoted more by the presence of Cu^{2+} than the original D-glucosamine hydrochloride, exhibits stronger radical signals in the ESR spectrum. It is suggested that D-glucosamine is unstable around neutral pH, being converted into certain intermediate(s) such as a dihydropyrazine compound, which generate(s) carbon-centered radicals, and that, besides the hydroxyl radical, the intermediate(s) is/are responsible for DNA strand breakage.

Key words D-glucosamine; DNA strand-breakage; dihydropyrazine; carbon-centered radical

Some carbohydrate derivatives have been shown to break DNA strands, especially in the presence of Cu^{2+} , and in some cases the oxygen radicals generated in aqueous solution have been demonstrated to be involved in this DNA strand breaking.¹⁾ The elucidation of the specific features of these DNA-breaking agents, particularly by comparison with those of DNA-alkylating or -intercalating compounds, will help clarify the mechanism of biological phenomena such as carcinogenesis, carcinostasis, mutagenesis, virus induction, virus inactivation, and aging. Furthermore, since fructosamine, an Amadori rearrangement²⁾ product formed by condensation of glucose and a protein amino group, has been shown to have the higher DNA-breaking activity than glucosamine, the action of aminosugars might be correlated with the caused mechanism of diabetes through radical formation.

In a previous paper,³⁾ we showed that some aminosugars broke plasmid pBR322 DNA, especially in the presence of Cu^{2+} , and that the coexistence of a potential free aldehyde group at the C-1 position and a free amino group at the C-2 position in the molecules were indispensable for DNA strand-breaking activity. Subsequently, the dehydrochlorinated product of D-glucosamine hydrochloride was shown to have much higher DNA-breaking activity than the original D-glucosamine hydrochloride.⁴⁾ These results suggested that a dihydropyrazine compound, which was identified on the basis of positive-ion FAB-MS as 2,5-bis(D-arabino-tetrahydroxybutyl)dihydropyrazine, might have been produced by condensation of two molecules of D-glucosamine and that the compound thus produced was responsible for DNA strand-breakage. This supposition was confirmed by the fact that dihydropyrazine derivatives synthesized independently showed DNA strand-breaking activity, and that the dihydropyrazine skeleton was essential for this.^{4,5)} Concerning the stimulating effect of Cu^{2+} on DNA strand-breakage, we have assumed that Cu^{2+} might be changed into Cu^{1+} by reaction with dihy-

dropyrazine, followed by transformation of dihydropyrazine into a carbon-centered radical.⁵⁾

Previously, the generation of superoxide anion, hydrogen peroxide, and hydroxyl radical from the aqueous solutions of various amino sugars was confirmed by direct measurement, and the hydroxyl radical finally formed was considered to be essential for inducing DNA strand-breakage.⁶⁾ However, DNA strand-breakage by D-glucosamine as well as its 6-phosphate occurred in the presence of Cu^{2+} in a sequence-specific manner, especially at the pyrimidine–purine (5'→3') site, but not uniformly at all nucleotide sites.⁷⁾ Therefore, certain carbon-centered radicals other than hydroxyl radical were suggested to be formed in the reaction mixture and involved in DNA strand-breakage. In fact, the formation of peroxy radicals in the presence of Cu^{2+} and their involvement in DNA strand-breakage have been suggested both for autoxidized lipids⁸⁾ and ascorbic acid,⁹⁾ and the formation of free radicals from monosaccharide has been shown by an electron spin resonance (ESR) spin trapping technique.^{10,11)}

In this paper, therefore, we have investigated the formation of carbon-centered radicals from D-glucosamine in Cu^{2+} -containing Tris–HCl buffered aqueous solutions by an ESR spin-trapping technique using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and sodium 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS).

MATERIALS AND METHODS

ESR Analysis ESR spectra were recorded with a JES-RE1X spectrometer (JEOL Co., Tokyo) using Mn^{2+} marker as an external standard. For ESR measurements of aqueous sample solutions, Quartz flat cell: LLC-04B (Labotec Co., Tokyo) was used. The instrumental conditions were: field set=335 mT, scan range=±5 mT, modulation frequency=100 kHz, field modulation width=1×0.1 (or 0.5×0.1) mT, time constant=0.1 s, receiver gain=4.0 (or 5.0, 10) ×100,

* To whom correspondence should be addressed.

microwave power=8 mW, and microwave frequency=9.41 GHz. The signals ($g_2=2.037$ and $g_1=1.981$) of the Mn^{2+} marker were recorded at the edges on both sides in all the spectra measured. The amino sugars (100 mM) were incubated in 50 mM Tris-HCl buffer (pH 7.1) at 37 °C for 1–8 h and, at intervals, 200 μ l-aliquots were taken and immediately stored at –30 °C until analyzed. The ESR spectra were recorded to observe a rapid change in signal pattern at 20 s of internal sweep time and to assign signals at 2 min of recording sweep time.

Chemicals D-Glucosamine hydrochloride, EDTA-2Na and cupric chloride dihydrate were obtained from Wako Pure Chemicals, LTD., Osaka. D-Mannosamine hydrochloride and the other sugars were from Nakalai Chemicals, LTD., Kyoto. Trizma Base was from Sigma Chemical Co., St. Louis, Missouri. 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) and sodium 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS) were from Labotec Co., Tokyo. HCl-free D-glucosamine sample⁴⁾ and 2,3-dihydro-5,6-dimethylpyrazine⁵⁾ were synthesized as described previously.

RESULTS AND DISCUSSION

Since it was presumed that DNA strand-breakage by D-glucosamine occurred when 2,5-bis(D-arabino-tetrahydroxybutyl)dihydropyrazine was produced from D-glucosamine, the solution of D-glucosamine hydrochloride (100 mM) in 50 mM Tris-HCl buffer (pH 7.1) was incubated at 37 °C for 0, 1, 3, 5, and 8 h. The ESR spectra of the solution were as shown in Fig. 1. Signals of DMPO- and DBNBS-adducts were detected. However, there was a remarkable difference between DMPO- and DBNBS-adduct signals. The signals of DBNBS-adducts were first detected at 3 h after incubation and then the intensity increased with time. The signals of

DMPO-adducts were detected immediately even without D-glucosamine hydrochloride (Fig. 1, a-1). The signal pattern and intensity of DMPO-adducts varied with the period of incubation, probably because DMPO itself reacted with Cu^{2+} to give radical signals due to DMPO before D-glucosamine reacted with Cu^{2+} . At 8 h after incubation (Fig. 1, a-6), the signal intensity of DMPO-adduct due to a carbon-centered radical dwarfed that due to hydroxyl radical. Therefore, the appearance of signals of DMPO-adducts due to D-glucosamine was considered to require a certain incubation time, more than 3 h. The ESR spectrum of D-glucosamine hydrochloride measured after 6 h of incubation was assigned to three component DMPO-adducts as shown in Fig. 2, a. Namely, four signals for the hydroxy radical component, which was characterized by the parameters; $g=2.0061$ and by the hyper-fine splitting constants (hfsc) of $aN=1.49$ mT and $aH=1.49$ mT, six signals for the carbon-centered radical component ($\bullet R$), which was characterized by $g=2.0061$ and hfsc of $aN=1.60$ mT and $aH=2.27$ mT (these signals were in agreement with that of the HCl-free D-glucosamine sample and 2,3-dihydro-5,6-dimethylpyrazine as described later and, these hfsc were supported by a previous paper¹²⁾), and three unidentified signals. The signal for the DBNBS-adducts (Fig. 3, a) of D-glucosamine hydrochloride could not be assigned from this spectrum, because the intensity was too weak to monitor any characteristics. The ESR spectra of D-glucosamine hydrochloride incubated with Cu^{2+} in the same buffer at 37 °C showed no DBNBS-adduct signals at any incubation time, probably because the radicals generated reacted with each other or were converted to a more stable form. The ESR spectra of the HCl-free D-glucosamine sample⁴⁾ obtained by the use of spin traps DMPO and DBNBS are shown in Fig. 2, b and Fig. 3, b, respectively. The HCl-free D-glucosamine sample having higher DNA strand-break-

Fig. 1. The ESR Spectra of D-Glucosamine Hydrochloride Incubated in 50 mM Tris-HCl Buffered Aqueous Solution (pH 7.1) at 37 °C for Various Times (0, 1, 3, 5, and 8 h)

The measured solution consists of 188 μ l incubated solution of D-glucosamine hydrochloride (100 mM), 2 μ l cupric chloride aqueous solution (1 mM), and 10 μ l DMPO or 10 μ l aqueous solution of DBNBS (20 mg/200 μ l). The charts of a-1 and b-1 were for the blank tests without D-glucosamine hydrochloride. The incubation time was 0 h for a-2 and b-2, 1 h for a-3 and b-3, 3 h for a-4 and b-4, 5 h for a-5 and b-5, and 8 h for a-6 and b-6, respectively. The ESR spectrum was recorded at 2 min using DMPO (upside) or at 5 min using DBNBS (downside) after mixing at room temperature; internal sweep time was 20 s, receiver gain was 10×100 , except for 5×100 (a-1 and a-2), field modulation width was 1×0.1 , time constant was 0.1.

Fig. 2. The ESR Spectra of DMPO-Adducts of D-Glucosamine Hydrochloride (a), HCl-Free D-Glucosamine Sample (b), and 2,3-Dihydro-5,6-dimethylpyrazine (c)

The measured solution consists of 2 μ l cupric chloride aqueous solution (1 mM), 10 μ l DMPO, and (a): 188 μ l incubated solution of D-glucosamine hydrochloride (100 mM) in 50 mM Tris-HCl buffer (pH 7.1) at 37 °C for 6 h, or (b): 20 μ l aqueous solution of HCl-free D-glucosamine sample (38.9 mg/100 μ l)+168 μ l 50 mM Tris-HCl buffer (pH 7.1), or (c): 20 μ l aqueous solution of 2,3-dihydro-5,6-dimethylpyrazine (3 μ l/500 μ l)+168 μ l 50 mM Tris-HCl buffer (pH 7.1). The ESR spectra were recorded at 2 min after mixing at room temperature; sweep time was 2 min, receiver gain was 5 $\times$ 100 for (a), 4 $\times$ 100 for (b) and (c), field modulation width was 0.5 $\times$ 0.1 for (a), 1 $\times$ 0.1 for (b) and (c), time constant was 0.1. The signals with * and + were the adducts of $\bullet$ OH and $\bullet$ R, respectively.

ing activity than D-glucosamine hydrochloride has been shown to have the dihydropyrazine compound as one of the intermediates. We have presumed that this sample was kept in similar conditions to the D-glucosamine hydrochloride incubated in buffer (pH 7.1). The spectrum (Fig. 2, b) of the HCl-free D-glucosamine sample showed two component DMPO-adducts: four signals due to a hydroxy radical and six signals due to a carbon-centered radical (as described previously). However, the signal (Fig. 3, b) of DBNBS-adducts of the HCl-free D-glucosamine sample could not be assigned as in the case of D-glucosamine hydrochloride. Figure 5 recorded at 20 s of internal sweep time showed the ESR spectra of the HCl-free D-glucosamine sample by using DMPO in the absence of Cu^{2+} at various times (2, 10, and 15 min) after mixing with EDTA-2Na and DMPO. The ESR spectrum (Fig. 5, b) at 10 min after mixing was assigned to three component DMPO-adducts; four signals for a hydroxyl radical component ($\bullet$ R) and three weak signals of an unidentified one. In the spectrum (Fig. 5, a) recorded at 20 s of internal sweep

Fig. 3. The ESR Spectra of DBNBS-Adducts of D-Glucosamine Hydrochloride (a), HCl-Free D-Glucosamine Sample (b), and 2,3-Dihydro-5,6-dimethylpyrazine (c)

The measured solution consists of 2 μ l cupric chloride aqueous solution (1 mM), 10 μ l aqueous solution of DBNBS (20 mg/200 μ l), and (a): 188 μ l incubated solution of D-glucosamine hydrochloride (100 mM) in 50 mM Tris-HCl buffer (pH 7.1) at 37 °C for 6 h, or (b): 20 μ l aqueous solution of HCl-free D-glucosamine sample (38.9 mg/100 μ l)+168 μ l 50 mM Tris-HCl buffer (pH 7.1), or (c): 20 μ l aqueous solution of 2,3-dihydro-5,6-dimethylpyrazine (3 μ l/500 μ l)+168 μ l 50 mM Tris-HCl buffer (pH 7.1). The ESR spectra were recorded at 5 min after mixing at room temperature; sweep time was 2 min, receiver gain was 10 $\times$ 100 for (a) and (b), 2 $\times$ 100 for (c), field modulation width was 0.5 $\times$ 0.1 for (a) and (b), 1 $\times$ 0.1 for (c), time constant was 0.1.

time, the four signals, whose intensities were stronger than the signals due to the hydroxyl radical, were assigned to be the signals of the DMPO-OOH adduct¹³⁾ by the parameters: $g=2.0061$, $aN=1.51$ mT, $aH\beta=1.12$ mT, $aH\gamma=0.13$ mT in the spectrum (Fig. 4, b) recorded at 2 min of sweep time. This signal pattern which was identified as the signals of $\bullet$ OH and $\bullet$ OOH in Fig. 4, b, was in good agreement with that (Fig. 4, a) of D-glucosamine hydrochloride. It is clear, therefore, that the generation of a carbon-centered radical was reduced by the presence of EDTA-2Na (Cu^{2+} was not added in this case), and that Cu^{2+} promoted the generation of carbon-centered radicals. The intensities of both the DMPO- and DBNBS-adduct signals of the HCl-free D-glucosamine sample gradually decreased with the passage of time at room temperature after mixing of the sample with Cu^{2+} (or EDTA-2Na) and trapping agent, and the signals had disappeared completely 15 min after mixing (Fig. 5, c). The characteristic difference was recognized by the fact that the signals due to DMPO itself reacting with Cu^{2+} in the absence of D-glucosamine maintained a strong intensity 15 min after mixing and then increased.

On the other hand, the ESR spectra of 2,3-dihydro-5,6-dimethylpyrazine⁵⁾ showed clear signals of both the DMPO-

Fig. 4. The ESR Spectra of DMPO-Adducts of D-Glucosamine Hydrochloride (a), HCl-Free D-Glucosamine Sample (b) in the Presence of EDTA-2Na (Cu^{2+} was not added)

The measured solution consists of 10 μl EDTA-2Na aqueous solution (100 mM), 10 μl DMPO, and (a): 180 μl incubated solution of D-glucosamine hydrochloride (100 mM) in 50 mM Tris-HCl buffer (pH 7.1) at 37°C for 6 h, or (b): 20 μl aqueous solution of HCl-free D-glucosamine sample (38.9 mg/100 μl)+160 μl 50 mM Tris-HCl buffer (pH 7.1). The ESR spectra were recorded at 2 min after mixing at room temperature; sweep time was 2 min, and receiver gain was 10×100 for (a), 5×100 for (b), field modulation width was 1×0.1 for (a), 0.5×0.1 for (b), time constant was 0.1. The signals with * and + were the adducts of $\cdot\text{OH}$ and $\cdot\text{OOH}$, respectively.

adducts (Fig. 2, c) and DBNBS-adducts (Fig. 3, c). This compound synthesized separately has been found to have a higher DNA strand-breaking activity, being promoted by the presence of Cu^{2+} . We have presumed that this compound having a dihydropyrazine skeleton might be one of the intermediates derived from D-glucosamine. The ESR spectra were assigned to two component DMPO-adducts (Fig. 2, c): four signals showing a hydroxyl radical ($\cdot\text{OH}$, $g=2.0061$; $a\text{N}=1.49\text{ mT}$ and $a\text{H}=1.49\text{ mT}$), six signals showing a carbon-centered radical ($\cdot\text{R}$, $g=2.0061$; $a\text{N}=1.60\text{ mT}$ and $a\text{H}=2.27\text{ mT}$), and a sole DBNBS-adduct component (Fig. 3, c): nine signals showing a carbon-centered radical ($\cdot\text{CH}_2\text{-R}$, $g=2.0066$; $a\text{N}=1.38\text{ mT}$ and $a\text{H}=1.01\text{ mT}$). In contrast to the ESR spectra of D-glucosamine, this ESR spectrum (Fig. 2, c) of DMPO-adducts showed that the intensity of the carbon-centered radical was higher than that of the hydroxyl radical. The hydroxyl radical detected here was considered to be due to oxygen dissolved in the reaction system because the intensity of the signal was reduced by bubbling argon gas through the solution. The signal intensities of both the adducts continued to increase even 30 min after mixing Cu^{2+} and trapping agent.

The ESR spectra of the signals of the DMPO-adduct of D-glucosamine hydrochloride, HCl-free D-glucosamine sample, and 2,3-dihydro-5,6-dimethylpyrazine were indistinguishable, and the signals indicated the presence of hydroxyl and carbon-centered radicals ($\cdot\text{R}$). It was presumed that the signals of the DBNBS-adduct of D-glucosamine hydrochloride and the HCl-free D-glucosamine sample may indicate the

Fig. 5. Variation of the DMPO-Adduct Signals of the HCl-Free D-Glucosamine Sample in the Absence of Cu^{2+}

The measured solution consists of 20 μl HCl-free D-glucosamine sample aqueous solution (38.9 mg/100 μl), 160 μl 50 mM Tris-HCl buffered aqueous solution (pH 7.1), 10 μl EDTA-2Na aqueous solution (100 mM), and 10 μl DMPO. The spectra were recorded at the passage time (2 min for a, 10 min for b, and 15 min for c) after mixing at room temperature; internal sweep time was 20 s, receiver gain was 5×100 , field modulation width was 1×0.1 , time constant was 0.1. The signals with * and + were the adducts of $\cdot\text{OH}$ and $\cdot\text{R}$, respectively.

presence of carbon-centered signals such as $\cdot\text{CH}_2\text{-R}$ while the others come from the intermediates of D-glucosamine. These results suggest that in the case of aminosugars like D-glucosamine having hydroxyl groups and a long carbon chain in their structure, the generation of various carbon-centered radicals together with hydroxyl radical might be predictable. Both D-mannosamine and D-galactosamine, incubated under the same conditions for 6 h, were also shown by the ESR spectra of the DBNBS-adducts to generate certain carbon-centered radicals. However, the signal patterns of the spectra of these aminosugars differed from each other in some details. The order of the signal intensity of the DBNBS-adducts of aminosugars was D-mannosamine > D-galactosamine > D-glucosamine, and this order agreed with their DNA strand-breaking activities. When N-acetyl D-glucosamine and N-acetyl D-mannosamine, which have no DNA strand-breaking activity, were incubated under the same conditions, no DBNBS-adduct signal was observed in their ESR spectra.

The ESR spectrum of D-glucosamine hydrochloride in a Tris-HCl buffer indicated that certain carbon-centered radicals from intermediates, including compounds having dihydropyrazine skeleton, were the essential reactive species together with a hydroxyl radical in the DNA strand-breakage reaction. It was suggested, therefore, that the carbon-centered radical might have played an important role in breaking the DNA strand in a sequence-specific manner. DNA strand-breakage by carbon-centered radicals generated in aqueous solution has been demonstrated with the hemoglobin- and peroxidase-catalyzed oxidation products of 2-phenylethylhydrazine and methylhydrazine,^{14,15} 5-diazouracil,¹⁶ and fluorene-2-diazonium salt.¹⁷ In the oxygen-derived radicals, the hydroxyl radical has been considered to abstract hydrogen atoms from the deoxyribose moiety of DNA to give initially 4'-carbon radicals, which lead to the breakage of the 3'-4' or 4'-5' bond and to the liberation of the phosphate group from the DNA backbone.¹⁸ The detailed mechanisms of DNA strand-breakage by carbon-centered radicals generated from D-glucosamine, remain to be elucidated. We are now investigating whether or not there is any specificity of the nucleotide sequence in dihydropyrazine derivative-induced

DNA strand-breakage.

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