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Comparison of Oxidation Products from DNA Components by γ-Irradiation and Fenton-Type Reactions

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The four 2'-deoxyribonucleosides were γ-irradiated or were aerobically treated with Fenton-type-reagents, Fe(II)-EDTA or a renal carcinogen Fe(II)-nitrilotriacetic acid (NTA) under the neutral conditions. The reaction mixtures were immediately analyzed by reverse-phase HPLC. Major products detected were 2-hydroxydeoxyadenosine (2-OH-dA), 8,5'-cyclodeoxyadenosine (cyclo-dA), 8-hydroxydeoxyadenosine (8-OH-dA), 5-formyldeoxyuridine (5-CHO-dU), 5-hydroxydeoxycytidine (5-OH-dC), 8-hydroxydeoxyguanosine (8-OH-dG), 8,5'-cyclodeoxyguanosine (cyclo-dG), and glyoxal and its adduct with dG. Ratio of these oxidized products were dramatically changed depending upon the agents used. For example, 2-OH-dA was a modified nucleoside produced most efficiently by Fe(II)-EDTA, while 5-CHO-dU and 5-OH-dC were the major products by the Fe(II)-NTA treatment and γ-irradiation, respectively. Glyoxal itself was estimated to be produced most frequently (13 folds of 8-OH-dG) when treated with Fe(II)-EDTA, but its formation was not detected by the treatment with Fe(II)-NTA or by γ-irradiation. 8-OH-dA was not produced by Fe-EDTA or Fe-NTA but was produced by γ-irradiation. In contrast, 2-OH-dA was not produced by γ-irradiation. These results suggest that triphosphates of 2-OH-dA, cyclo-dA, 8-OH-dA, cyclo-dG, 5-CHO-dU, 5-OH-dC, and glyoxal-dG as well as 8-OH-dG may be produced in cells with different ratio by various types of oxidative stress and involved in mutagenesis and carcinogenesis.

INTRODUCTION

Oxidative stress have been shown to be involved in biological processes such as mutagenesis, carcinogenesis and ageing[1–3]. Reactive oxygen species (ROS) produced in cells react with DNA and its

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Abbreviations: ROS, Reactive oxygen species; 2-OH-dA, 2-hydroxydeoxyadenosine; cyclo-dA, 8,5'-cyclodeoxyadenosine; 8-OH-dA, 8-hydroxydeoxyadenosine; 5-CHO-dU, 5-formyldeoxyuridine; 5-OH-dC, 5-hydroxydeoxycytidine; 8-OH-dG, 8-hydroxydeoxyguanosine; cyclo-dG, 8,5'-cyclodeoxyguanosine; NTA, nitrilotriacetic acid.

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precursors, and the oxidative DNA lesions formed will cause mutational events. One of the oxidative DNA lesions is 8-hydroxydeoxyguanosine (8-OH-dG; 7,8-dihydro-8-oxodeoxyguanosine)[4-7] and it pairs with dA as well as dC in *in vitro* DNA synthesis and induces mainly G $\rightarrow$ T transversions in cells[8-16]. Moreover, 2-hydroxydeoxyadenosine (2-OH-dA) and 5-hydroxydeoxycytidine (5-OH-dC), which are produced by ROS, are miscoding and mutagenic[17-21].

Although various types of oxidative DNA damage have been extensively studied quantitatively and qualitatively, comparative study on their formation by different agents in monomer nucleosides has not been reported yet. Accumulated evidences indicate that oxidative damage in DNA precursors is as important as that produced in DNA, because DNA polymerases incorporate damaged monomers into DNA[12,22-24]. Moreover, loss of activity for hydrolyzing a damaged nucleoside 5'-triphosphate induces increase in mutation frequency[22]. Therefore, it is important to know modified products of monomers by treatment with ROS when we consider mutational processes which are caused by oxidatively damaged nucleotides. In this study, the four deoxyribonucleosides were aerobically treated with Fe(II)-EDTA, and a renal carcinogen[25], Fe(II)-nitrilotriacetic acid (NTA), which generate ROS by Fenton-type reactions[26], or were irradiated with γ-rays, in a neutral buffer solution as model systems of oxidation of DNA precursors in cells, and compared the yields of major products, 2-hydroxydeoxyadenosine (2-OH-dA), 8,5'-cyclodeoxyadenosine (cyclo-dA), 8-hydroxydeoxyadenosine (8-OH-dA), 5-formyldeoxyuridine (5-CHO-dU), 5-hydroxydeoxycytidine (5-OH-dC), 8-hydroxydeoxyguanosine (8-OH-dG), 8,5'-cyclodeoxyguanosine (cyclo-dG), and glyoxal and its adduct with dG (Fig. 1).

MATERIALS AND METHODS

Materials.

2'-Deoxyadenosine, 2'-deoxycytidine•HCl and thymidine were purchased from Yuki Gousei Kogyo Yakuhin Co. 2'-Deoxyguanosine was obtained from Wako Pure Chemical Industries Co. Glyoxal was from Nacalai Tesque Inc.

Preparation of Standard Samples.

2-OH-dA was synthesized by the Method of Kazimierczuk *et al*[27]. 8-OH-dG and 5-CHO-dU were synthesized as described previously[6,28]. 5-OH-dC was prepared according to the method of Eaton and Hutchinson for the riboside[29]. Glyoxal-dG was prepared by treatment of dG with 100-fold excess molar of glyoxal in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 30 min. Cyclo-dA and cyclo-dG were produced by the γ-irradiation of dA and dG[29,30] as described below. 8-OH-dA was a gift from Drs. Eiko Ohtsuka and Hideo Inoue.

Treatment of Deoxyribonucleoside with Fe(II)-EDTA and Fe(II)-NTA and HPLC Analysis.

A single deoxyribonucleoside (1 mM) or mixture of the four nucleosides (0.25 mM each) dissolved in 50 mM sodium phosphate buffer (pH 7.0) was treated with Fe(II)-EDTA (5 mM $FeSO_4$ and 5 mM EDTA) or Fe(II)-NTA (5 mM $FeSO_4$ and 20 mM NTA) at 37°C for 30 min. When 2'-deoxycytidine · HCl was used, its solution was first neutralized with NaOH and then the phosphate buffer was added. The $FeSO_4$ solution was prepared immediately before adding to other components. No pH change was observed before and after

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the incubation.

After completion of the incubation, the reaction mixture was injected immediately into a reverse-phase HPLC column [YMC-pack ODS-AM303 (4.6 × 250 mm)] connected with a photo-diode array UV detector Hewlett Packard 1100 HPLC Detection System. The following linear gradient of methanol concentrations in 10 mM NaH_2PO_4 were used: 0 min to 10 min, 0% methanol; 10 min to 30 min, linear gradient of methanol (0% to 5%); 30 min to 45 min, linear gradient of methanol (5% to 20%); 45 min to 48 min, 20% methanol. The flow rate was 1.0 ml/min.

Irradiation of γ-rays.

Irradiations of the four deoxyribonucleoside mixture (1 mM each) in 50 mM sodium phosphate buffer (pH 7.0) were carried out with a Gammacell 220 ^{60}Co γ-ray source (Atomic Energy of Canada) at a dose rate of 98 Gy/min (total 2900 Gy). Analysis by HPLC was conducted as described above.

RESULTS

Treatment of Deoxyribonucleoside with Fe(II)-EDTA.

When dA was treated with Fe(II)-EDTA, two major deoxyribonucleoside peaks other than starting materials were detected (Fig. 2A). We identified these materials as cyclo-dA (3) and 2-OH-dA (1) (Fig. 1)

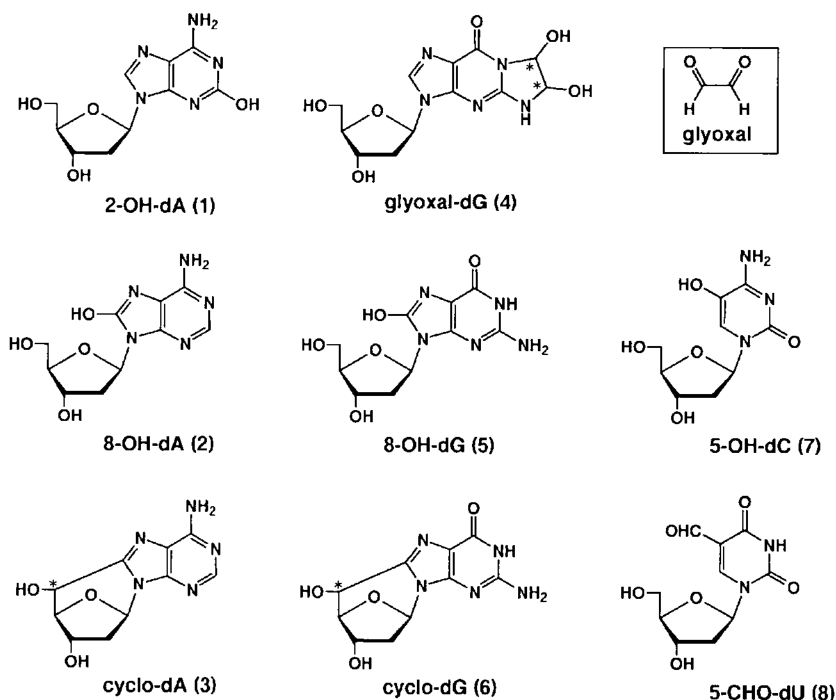

Fig. 1. The eight major modified compounds detected in this study. The asterisks on the carbon atoms of glyoxal-dG, cyclo-dA and cyclo-dG show chiral centers. The structure of glyoxal is also shown (in square).

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by comparison of their chromatographic behaviors and UV-spectra with those of standard samples (Fig. 2). In the reaction mixture of dG, glyoxal-dG adduct (4,4'), cyclo-dG (6) and 8-OH-dG (5) were found (Fig. 2B). In the cases of dC and dT, 5-OH-dC (7) and 5-CHO-dU (8) were identified, respectively (Fig. 3). These assignments were also made by their chromatographic and spectral properties (Figs. 2 and 3).

Four stereoisomers of glyoxal-dG and two stereoisomers of the cyclo compounds are possible due to the chiralities of carbon atoms in modified nucleosides (Fig. 1). However, we observed the two peaks for glyoxal-dG (4, 4') and single peaks for the cyclo compounds (3,6) under the conditions used (Fig. 2A and B). It is not clear whether separation of the isomers was inefficient or only two (glyoxal-dG) or one (the cyclo compounds) isomer(s) were formed. Cyclo-dG (6) was co-eluted with "slow isomer(s)" of glyoxal-dG (4') (Fig. 2B). We estimated the yield of both compounds by assuming that the ratio of areas of "fast" to "slow" isomers of glyoxal-dG were constant.

Table 1 shows yields of each compound under the same reaction conditions {5 mM Fe(II)-EDTA, 37°C, 30 min}. The reactions and analyses were conducted at least in duplicate and good reproducibilities were obtained. The yields were shown as percent of a modified nucleoside versus total (modified plus

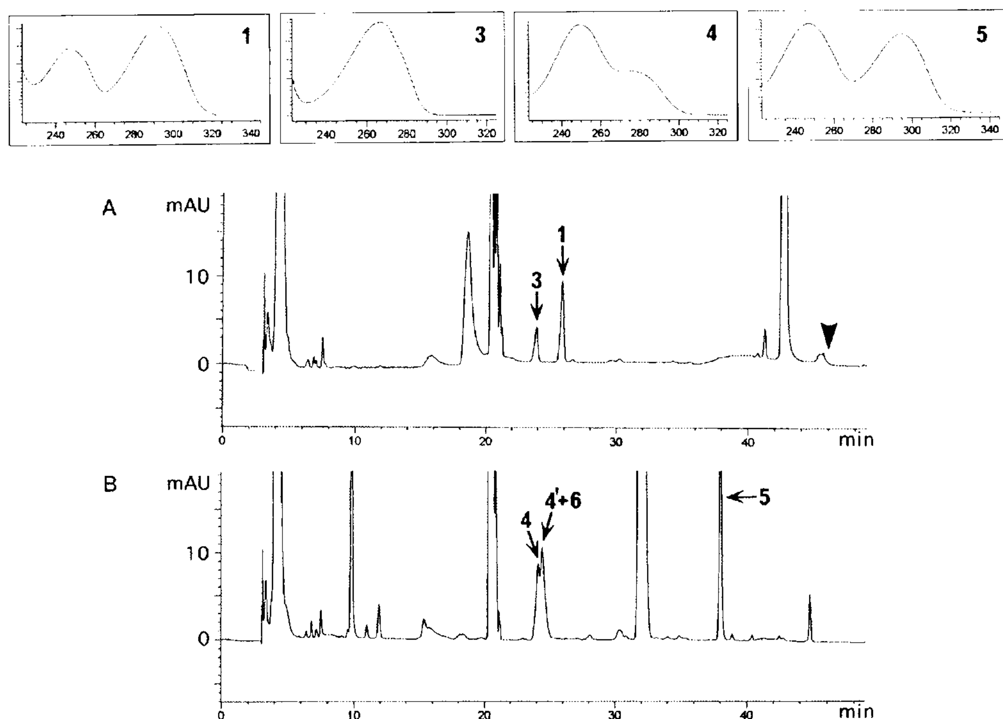

Fig. 2. HPLC analysis of the reaction mixtures of (A) dA and (B) dG treated with Fe(II)-EDTA. Reactions were carried out according to the Materials and Method section. Elution conditions used were followings. 0 min to 10 min, 0% methanol; 10 min to 30 min, linear gradient of methanol (0% to 5%); 30 min to 45 min, linear gradient of methanol (5% to 20%); 45 min to 48 min, 20% methanol. Numbers which indicate each peak correspond to those in Fig. 1. An arrowhead in panel A indicates the position where 8-OH-dA is eluted. Detection was performed with UV absorbance at 256 nm. UV spectra of peaks 1, 3, 4, and 5 are shown above.

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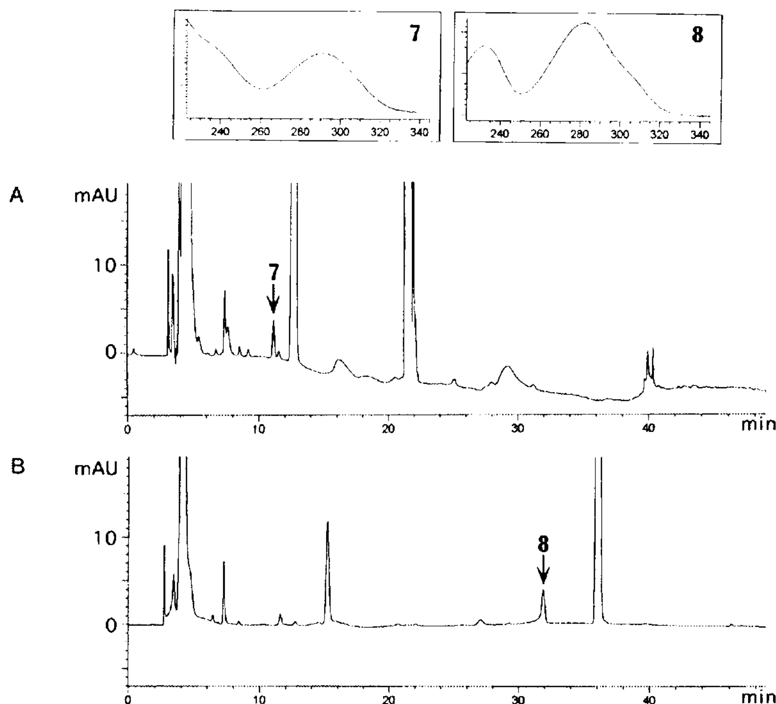

Fig. 3. HPLC analysis of the reaction mixtures of (A) dC and (B) dT treated with Fe(II)-EDTA. Elution conditions used were same as those described in the legend for Fig. 2. Numbers which indicate each peak correspond to those in Fig. 1. Detection was performed with UV absorbance at 256 nm. UV spectra of peaks 7 and 8 are shown above.

unmodified) nucleosides. Yields of each product increased depending on the concentration of Fe(II)-EDTA (1–5 mM, data not shown). The major modified nucleoside was 2-OH-dA and it was generated 1.8-fold more than 8-OH-dG. 5-CHO-dU, 5-OH-dC and glyoxal-dG were formed as much as 8-OH-dG. The seven compounds were produced in the order of 2-OH-dA > 5-CHO-dU > 8-OH-dG > 5-OH-dC > glyoxal-dG > cyclo-dG > cyclo-dA (Table 1). These results suggest that 2-OH-dA, 5-CHO-dU, 5-OH-dC, glyoxal-dG, cyclo-dG and cyclo-dA should be focused on as well as 8-OH-dG when we consider damage of DNA precursors by ROS. Formation of 8-OH-dA was not observed (Fig. 2A).

It should be noted that glyoxal-dG is formed by the reaction of the intact dG remained and glyoxal which is generated from dG by the Fe(II)-EDTA treatment. Therefore, the net amount of glyoxal generated will be more than that of its dG-adduct. We treated dG with different amounts of glyoxal and measured formation of the glyoxal-dG adduct as a function of amounts of glyoxal (data not shown). We then estimated that about 8% of the original dG was converted to glyoxal by the calculation from the amount of the glyoxal-dG adduct formed in the Fe(II)-EDTA reaction of dG. This value is 3.2 folds of that of 8-OH-dG when dG was treated. Therefore, if the equal amount mixture of the four deoxynucleosides is treated, glyoxal will be produced about 13-fold more than 8-OH-dG under the conditions used because glyoxal is formed from other monomers without base-selectivity[32].

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Treatment of Deoxyribonucleoside with Fe(II)-NTA.

With Fe(II)-NTA, the same products except for glyoxal-dG were detected (Fig. 4). The major modified nucleoside produced was 5-CHO-dU (3.1 folds of 8-OH-dG, Table 1). 2-OH-dA and 5-OH-dC were formed with 0.6-fold yields of 8-OH-dG. The cyclo compounds were generated as much as 8-OH-dG. Therefore, oxidized nucleosides were formed by Fe(II)-NTA in the order of 5-CHO-dU > cyclo-dA > 8-OH-dG ~ cyclo-dG > 2-OH-dA ~ 5-OH-dC with Fe(II)-NTA (Table 1). Similar results were obtained when a mixture of equall amounts of the four nucleosides was treated. Amounts of 2-OH-dA, 8-OH-dG and 5-OH-dC were less than 1/3 of those with Fe(II)-EDTA (Table 1). Particularly, the formation of 2-OH-dA with Fe(II)-NTA was less than 1/10 of that with Fe(II)-EDTA. In contrast, 5-CHO-dU was produced by Fe(II)-NTA with a yield of 70% of the Fe(II)-EDTA treatment. The cyclo compounds were produced with similar efficiencies to the Fe(II)-EDTA treatment. We detected no peak corresponding to glyoxal-dG. No contamination of glyoxal-dG around the cyclo-dG peak was estimated by UV spectrum of the peak when dG only was treated with Fe(II)-NTA (data not shown). Failure to detect glyoxal by the orthophenylenediamine method[32] also indicated that little, if any, glyoxal was generated by Fe(II)-NTA (data not shown). The peak corresponding to 8-OH-dA was not observed (Fig. 4).

Treatment of Deoxyribonucleoside with γ-rays.

After irradiation of γ-rays to the deoxyribonucleoside mixture, formation of 5-OH-dC, cyclo-dA, cyclo-dG, CHO-dU, 8-OH-dG and 8-OH-dA was observed (Fig. 5). It should be noted that 2-OH-dA was not formed. It was contrast to the cases of Fe(II)-EDTA and Fe(II)-NTA with which 2-OH-dA was formed (Figs. 2A and 4). Glyoxal-dG was not detected as Fe(II)-NTA. Failure to detect glyoxal by the orthophenylenediamine method[32] also indicated that little, if any, glyoxal was generated by γ-irradiation

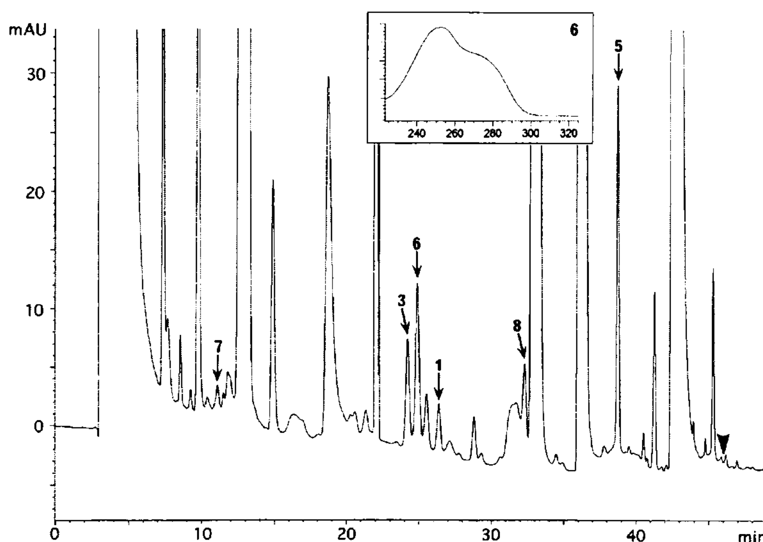

Fig. 4. HPLC analysis of the reaction mixtures of the four deoxyribonucleosides treated with Fe(II)-NTA. Numbers which indicate each peak correspond to those in Fig. 1. An arrowhead indicates the position where 8-OH-dA is eluted. Detection was performed with UV absorbance at 256 nm. UV spectrum of peak 6 is shown (inset).

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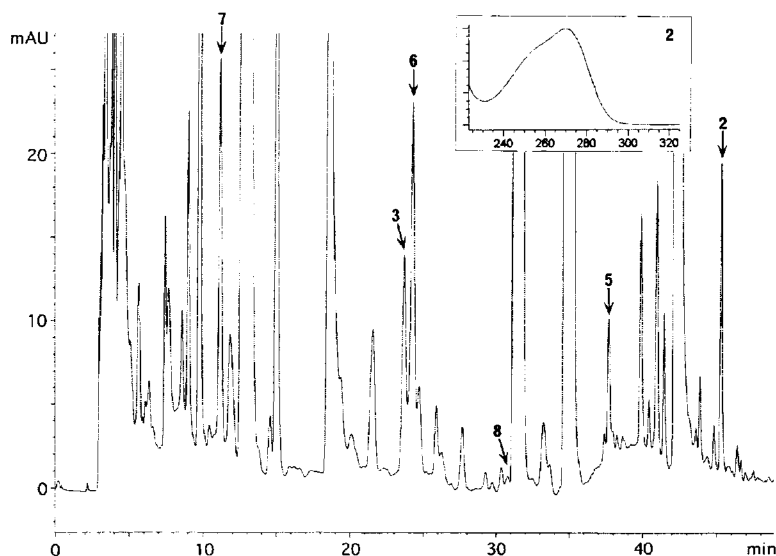

Fig. 5. HPLC analysis of the mixtures of the four deoxyribonucleosides irradiated with γ-ray. Numbers which indicate each peak correspond to those in Fig. 1. Detection was performed with UV absorbance at 256 nm. UV spectrum of peak 2 is shown (inset).

(data not shown).

DISCUSSION

In this study we attempted to compare various oxidative products of DNA components by Fe(II) ion auto-oxidation systems and γ-rays as a source of ROS. We observed different ratio of oxidized products by γ-irradiation, Fe(II)-EDTA and Fe(II)-NTA treatments (Table 1). These results suggest that ROS generated by these three agents are not identical. It is also possible that tertiary structures of DNA-Fe-EDTA and DNA-Fe-NTA complexes are different when an oxidation reaction occurs.

2-OH-dA was a modified nucleoside produced most efficiently by Fe(II)-EDTA but its formation with Fe(II)-NTA was less than 1/10 of Fe(II)-EDTA (Table 1). Although we attempted to investigate whether 8-OH-dA, which is known to be produced by γ-irradiation[33], was formed with Fe(II) ion auto-oxidation systems, no corresponding peak was observed with either Fe(II)-EDTA (Fig. 2A) or Fe(II)-NTA (Fig. 4). Because we detected 8-OH-dA in γ-irradiated solution of dA or the four nucleoside mixture (Fig. 5), we concluded that 8-OH-dA was not produced from dA by treatment with an auto-oxidation system of Fe(II) ions at least under the conditions used in this study. Interestingly, 2-OH-dA was not produced by γ-irradiation. Thus, the hydroxylation position in the adenine base is dependent upon the kind of ROS-generating systems.

Glyoxal was estimated to be produced most efficiently with Fe(II)-EDTA. About 3.2 folds of glyoxal was estimated to be produced compared with 8-OH-dG when dG was treated. Therefore, glyoxal will be produced about 13-fold more than 8-OH-dG in the four nucleoside mixture under the conditions used. On

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the other hand, formation of glyoxal was not detected with Fe(II)-NTA *in vitro*. Therefore, this oxidative product may not be involved in NTA-induced carcinogenesis although the involvement of ROS in the process is assumed[34–36].

5-CHO-dU was the major products in the Fe(II)-NTA treatment. Its yields in both Fe(II)-EDTA and -NTA treatments were similar (Table 1). In contrast, its formation by γ-irradiation seemed to be much less efficient when compared with cyclo-dA, cyclo-dG, or 8-OH-dG. 5-OH-dC, the product formed most efficiently with γ-irradiation appeared to be produced less efficiently with Fe(II)-NTA (Table 1).

It has been reported that cyclo-dA and cyclo-dG are produced by γ-irradiation of dA and dG, respectively[30,31]. We observed the formation of the cyclo compounds in the reactions of autooxidation of Fe(II) ion. The formation of cyclo-dA from dA by these reactions has never been reported although production of cyclo-dG by the Fenton reaction was recently reported[37].

Of the products described above, 2-OH-dA, 8-OH-dA, 8-OH-dG and 5-OH-dC in DNA are miscoding *in vitro* and mutagenic in cells[8–21,38]. Moreover, their corresponding nucleotides (deoxynucleoside 5'-triphosphates) are incorporated into a growing DNA strand by DNA polymerases[12,22–24]. Therefore, the formation of the damaged DNA precursors in the nucleotide pool will induce mutations. Moreover, 5-CHO-dU is shown to be mutagenic in the Ames test using *Salmonella typhymurium* strain TA102[28] indicating that the nucleoside, after the conversion to the cognate 5'-triphosphate, is incorporated into DNA and induces mutations.

Although it is unknown whether glyoxal-adducted dGTP is incorporated into DNA and induces mutation, glyoxal itself is a mutagen[32] and induces point mutations in *S. typhymurium* and *Escherichia coli*[39,40]. Probably glyoxal reacts with DNA and/or dGTP and then induces mispairing observed in those studies. Moreover, miscoding properties of cyclo-dA and cyclo-dG have not reported and remain to be resolved.

In this paper we showed that glyoxal, 2-OH-dA, 5-CHO-dU, 5-OH-dC, cyclo-dA, cyclo-dG, and 8-

Table 1. Yields of modified nucleosides formed by ROS[a]

Product	Treatment		
	Fe-EDTA[b]	Fe-NTA[b]	γ-ray[c]
2-OH-dA	4.8 (1.8)	0.4 (0.6)	ND[d]
cyclo-dA	0.8 (0.3)	0.8 (1.1)	0.6 (1.2)
8-OH-dA	ND	ND	0.6 (1.2)
8-OH-dG	2.6 (1.0)	0.7 (1.0)	0.5 (1.0)
glyoxal-dG	2.1 (0.8)	ND	ND
cycol-gG	0.9 (0.3)	0.7 (1.0)	0.7 (1.4)
5-CHO-dU	3.1 (1.2)	2.2 (3.1)	0.2 (0.4)
5-OH-dC	2.3 (0.9)	0.4 (0.6)	2.6 (5.2)

[a] Yields shown are the ratios (%) of a modified nucleoside to the sum of the modified and unmodified nucleosides. For modified dA and dG, all modified products are included in the calculations. The number in parentheses represents the yields relative to 8-OH-dG.

[b] A single deoxyribonucleoside was treated.

[c] A mixture of equall amounts of the four nucleosides was treated.

[d] Not detected.

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OH-dA as well as 8-OH-dG were formed with different ratios depending upon the ROS-generating systems used. Therefore, the 'origin' of mutations induced by ROS may differ in each case. It is noteworthy that 8-OH-dG was not the product formed most frequently in any of the three treatments. Further biological studies on these oxidized products including mutagenesis and repair studies[41)] will reveal the actual mechanism of ROS-related carcinogenesis processes.

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