Spin-Trapping Detection of Precursors of Hydroxyl-Radical-Induced DNA Damage: Identification of Precursor Radicals of DNA Strand Breaks in Oligo(dC)$_{10}$ and Oligo(dT)$_{10}$†

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ABSTRACT: A spin-trapping method combined with enzymatic digestion and high-performance liquid chromatography was employed to detect hydroxyl-radical-induced precursors of strand breaks in oligonucleotides ((dC)$_{10}$ and (dT)$_{10}$) as DNA models. Radicals produced as precursors of both strand breaks and base alterations were first trapped by the spin trap 2-methyl-2-nitrosopropane. The oligonucleotides containing spin adducts were subsequently digested by snake venom phosphodiesterase to release low-molecular-weight nitroxide fragments. In this way, several spin adducts were separated by high-performance liquid chromatography. In both oligonucleotides, ESR spectra attributable to the spin adducts derived from trapping of a precursor radical of strand breaks (the C4′-sugar radical) were observed. To further confirm this assignment, the induction of strand breaks was examined by polyacrylamide gel electrophoresis of 5′-^{32}P-end-labeled oligonucleotides. Autoradiograms of the gels showed that the fragments corresponding to monomers to 9mers were formed in both oligonucleotides. When experiments were carried out under conditions in which hydroxyl radicals reacted with oligomers in the presence of the spin trap, the spin trap was found to suppress the fragmentation more than it did by scavenging hydroxyl radicals, indicating that the precursor radical of strand breaks (the C4′ radical) was trapped. The present experiments showed that the spin-trapping method combined with gel electrophoresis was a good approach to identify sites of radical damage which cause strand breaks in oligonucleotides (probably in DNA).

Hydroxyl (OH) radicals, which are the most harmful among reactive oxygens, are known to be produced by physical, chemical, and biochemical sources (Sies, 1986; Byczkowski & Gessner, 1988). OH radicals react with biologically important molecules such as DNA, RNA, proteins, lipids, and polysaccharides to produce oxidative stress. OH radicals react with DNA to induce base alterations, abasic sites, and strand breaks which are thought to be strongly related to the serious effects on the cells (Chapman & Gillespie, 1981; von Sonntag, 1987). OH-radical-induced base or sugar radicals are regarded as reactive intermediates causing those base alterations, abasic sites, and strand breaks (von Sonntag, 1980; von Sonntag & Schuchmann, 1986). Although evidence for the generation of these radicals was obtained by employing purine and pyrimidine nucleoside monophosphates as model compounds of DNA and by observing free radicals formed in them with a method combining ESR,[1] spin trapping, and HPLC (Inanami et al., 1986, 1987; Kuwabara et al., 1989; Hiraoka et al., 1989, 1990; Flitter & Mason, 1989), the application of this method to polymerized forms of nucleoside monophosphates such as oligonucleotides is more desirable to approach actual DNA. When we applied the spin-trapping method to calf thymus DNA, we obtained evidence for the formation of precursor radicals of base alterations (Kuwabara et al., 1987). Recently, we improved the usual spin-trapping method by combining it with enzymatic digestion and HPLC and could successfully apply it to oligomers of RNA constituents, poly(U) (Inanami et al., 1987), poly(I) and poly(A) (Kuwabara et al., 1986), and poly(C) (Hiraoka et al., 1990), to get evidence for the formation of strand-break precursors. In the present study, we extended this method to the oligonucleotides of DNA constituents, oligo(dC)$_{10}$ and oligo(dT)$_{10}$, to more closely approach actual DNA. These pyrimidine oligonucleotides were chosen as samples because basic information about their monomers, TMP and dCMP, had already been reported in our previous studies (Kuwabara et al., 1989; Hiraoka et al., 1989), and they have the property of high solubility in aqueous solution, which was advantageous for spin-trapping experiments.

To obtain evidence that the assigned radical was, in fact, the precursor radical of strand breaks, we analyzed 5′-^{32}P-end-labeled oligomers, which were attacked by OH radicals under conditions in which the spin trap MNP was present or absent, with PAGE. In this manner, the analysis of PAGE patterns of oligomers was carried out to determine whether MNP suppressed the fragmentation of oligomers due to OH radicals by trapping the precursor radicals of strand breaks. In the present study, it was proved that a method combining spin trapping and gel electrophoresis was a good approach to clarify the relationship between OH-radical-induced sugar radicals and the strand breaks of oligomers.

EXPERIMENTAL PROCEDURES

Materials.

Decamers of 2′-deoxycytidine monophosphate, oligo(dC)$_{10}$, and of thymidine monophosphate, oligo(dT)$_{10}$, were synthe-

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[1] Abbreviations: dCMP, 2′-deoxycytidine 5′-monophosphate; ESR, electron spin resonance; HPLC, high-performance liquid chromatography; kGy, kilogray; MNP, 2-methyl-2-nitrosopropane; OH, hydroxyl; PAGE, polyacrylamide gel electrophoresis, dThd, thymidine; TMP, thymidine 5′-monophosphate; TBE, Tris–borate EDTA; TEA, triethylamine/acetic acid.

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sized with Cyclone Plus DNA Synthesizer from MilliGen/Biosearch Company (Massachusetts) according to the β-cyanoethyl phosphoramidite method. After the synthesized oligomers were treated with 28% NH_4OH at room temperature for 36 h to remove their support and to deprotect the phosphorous, they were purified stepwise by HPLC using a Tosoh CCP&8000 system (Tosoh Company, Tokyo, Japan). The first purification was carried out with a TSKgel OligoDNA RP reverse-phase column (4.6 mm i.d., 15 cm long, Tosoh Company) by eluting the solution containing oligomers with a 30-min linear gradient from 0.1 M TEA (pH 6.9)/CH_3CN (95:5, v/v) to 0.1 M TEA/CH_3CN (50:50, v/v) at a flow rate of 1 mL/min. After the solution was dried, the oligomers were treated with 80% acetic acid for 20 min at room temperature to remove dimethoxytrityl. After acetic acid was removed by drying, the second purification was carried out with a TSKgel DEAE-2SW ion-exchange column (4.6 mm i.d., 25 cm long) by eluting it with a 20-min linear gradient from a mixed solution (80:20, v/v) of (A) 20% aqueous solution of CH_3CN and (B) 2 M $HCOONH_4$ in 20% aqueous solution of CH_3CN to A/B (60:40, v/v) at a flow rate of 1 mL/min. The final purification was carried out with TSKgel OligoDNA RP by eluting it with a 30-min linear gradient from 0.1 M TEA (pH 6.9)/CH_3CN (95:5, v/v) to 0.1 M TEA/CH_3CN (50:50, v/v) at a flow rate of 1 mL/min. The purified oligomers were dried by centrifugal evaporation and again dissolved in triply distilled water at the appropriate concentrations for experiments.

Phosphodiesterase from *Crotalus durissus* (EC 3.1.15.1; 3 units/mL) was purchased from Boehringer Mannheim (Mannheim, Germany). T4 polynucleotide kinase from *Escherichia coli* 594NM575 (EC 2.7.1.78; 10 units/μL) was from Toyobo Company (Tokyo, Japan). The spin trap 2-methyl-2-nitrosopropane (MNP) was obtained from Aldrich Chemical Company Inc. (Milwaukee, Wi). [γ-^{32}P]ATP triethylammonium salt (4000 Ci/mmol) was from Bresatec Ltd. (Australia).

Methods.

Spin Trapping of OH-Radical-Induced Free Radicals in Oligomers. Aqueous solution containing 2 mg of oligo(dC)$_{10}$ or oligo(dT)$_{10}$ was first prepared with 1 mL of triply distilled water. MNP powder (1.3 mg) was put into the solution, which was then bubbled with N_2O gas for 15 min and sealed in a Pyrex tube. The solution was stirred overnight at room temperature in the dark to completely dissolve MNP powder and to achieve stable monomer–dimer equilibrium (Riesz & Rustgi, 1979; Kuwabara et al., 1991). The solution was exposed to 2.7 kGy of X-rays with a Toshiba X-ray machine at 180 kV and 25 mA. X-ray irradiation of the N_2O-saturated aqueous solution with a dose of 2.7 kGy generates 0.76 mM OH radicals (Kuwabara et al., 1989).

Enzymatic Digestion of Spin Adducts Derived from Oligomers. Oligomers containing spin adducts were enzymatically digested to low-molecular-weight nitroxide fragments prior to the separation with HPLC. Eighty microliters of aqueous solution containing 50 mM $MgCl_2$, 200 mM sodium acetate, pH 6.5, and 80 μL of phosphodiesterase solution was added to the X-ray irradiated aqueous solution containing oligomers. The solution was incubated at 37 °C for 1 h and transferred to a centrifugal filtration tube (UFC3LGC00, Nihon Millipore Ltd., Tokyo, Japan). The tube was then centrifuged at 55 000 rpm for 20 min at room temperature to remove the enzyme.

Separation of Spin Adducts by Reverse-Phase HPLC in the Ion-Suppression Mode. After 0.2 mL of 0.5 M TEA (pH 6.5) was added to the digested solution, the solution was injected into a TSKgel OligoDNA RP reverse-phase column attached to a Tosoh CCP&8000 HPLC system. TEA was used as a paired-ion reagent for the phosphate group (Ip et al., 1985). Before the solution was eluted with HPLC, the eluents, (A) 0.1 M TEA (pH 6.5) solution and (B) 1:1 (v/v) mixed solution of TEA (pH 6.5) and CH_3CN, were prepared. Elution was carried out with a mixture of A and B at a ratio of 98:2 for 5 min, followed by a 35-min linear gradient from a 98:2 (v/v) mixture of A and B to B at a flow rate of 0.5 mL/min. The eluted solution was monitored for UV absorbance at 260 nm with 1.28 AUFS. The presence of the spin adduct in each fraction was ascertained by ESR spectrometry.

Measurements of ESR Spectra. The separated spin adducts were characterized by ESR spectrometry. ESR measurements were made on a JEOL ME-1X X-band spectrometer (Akishima, Japan). The ESR spectra were recorded as first derivatives at room temperature. The ESR scans were traced with a 100-kHz field modulation of 0.02 mT amplitude, and the microwave power level was maintained at 20 mW. The α, β, and γ positions of magnetic nuclei in the spin adduct were determined with respect to the unpaired electron of the nitrogen of the nitroxide (Riesz & Rustgi, 1979).

5′-^{32}P-End-Labeling of Oligomers. Labeling of oligo(dC)$_{10}$ and oligo(dT)$_{10}$ at 5′ termini by a phosphate-exchange reaction with T4 polynucleotide kinase was made essentially according to the method of Maxam and Gilbert (1980). After aqueous solution containing 0.01 OD_{260nm} unit of oligonucleotide was dried by centrifugal evaporation, 1 μL of 250 mM Tris–HCl (pH 7.7) containing 50 mM $MgCl_2$, 1 μL of 50 mM 2-mercaptoethanol, 5.5 μL of H_2O, 2 μL of [γ-^{32}P]ATP, and 0.5 μL of T4 polynucleotide kinase were added to the dried sample. The solution was incubated at 37 °C for 1 h and further incubated at 90 °C for 5 min. To purify the labeled oligomer, this solution was first examined by 19% PAGE containing urea (see details of the method below). The X-ray film (Eastman Kodak, Colorado) was then exposed to β-rays from the gel plate, and a single spot of the gel containing 10mers was taken out of the plate, transferred into 1.5-mL Eppendorf snap-cap tubes containing 1 mL of 0.1 M TEA, and homogenized by shaking for 30 min. The homogenized solution was centrifuged for 10–20 s. The supernatant liquid was dried by centrifugal evaporation. The dried sample was dissolved in 0.025 M TEA, and the solution was filtered with a NAP-10 (Sephadex G25) column (Pharmacia, Uppsala, Sweden), by using 25 mM TEA buffer as an eluent to remove urea. The eluted solutions were dried by centrifugal evaporation, and the dried samples were dissolved in 50 μL of triply distilled water in Eppendorf snap-cap tubes.

Treatment of 5′-End-Labeled Oligo(dC)$_{10}$ and Oligo(dT)$_{10}$ with OH Radicals in the Absence of MNP for PAGE Analysis. An aqueous solution containing ^{32}P-oligomer was mixed with 950 μL of aqueous solution containing nonlabeled oligomers in Eppendorf snap-cap tubes so that OD_{260nm} became 0.3. The solution was subdivided into eight Eppendorf snap-cap tubes, and each was saturated with N_2O gas by bubbling the solution and sealed. On a Toshiba X-ray machine at 180 kV and 25 mA, X-ray irradiation was carried out at room temperature to produce OH radicals at a rate of 54 μM OH radicals/min.

Treatment of 5′-End-Labeled Oligo(dC)$_{10}$ and Oligo(dT)$_{10}$ with OH Radicals in the Presence of MNP for PAGE Analysis. Argon-saturated solution containing 0.2 mg of MNP was first prepared with 1 mL of triply distilled water by stirring for 12 h. A mixture of labeled and nonlabeled oligomers was then dissolved in a part of this solution so that OD_{260nm} became

0.31 and 0.39 for oligo(dC)$_{10}$ and oligo(dT)$_{10}$, respectively. The solution was saturated with N_2O gas by bubbling. Since this treatment completely removed the monomer of MNP, the solution was left at room temperature for 3 h to regain monomer–dimer equilibrium (Riesz & Rustgi, 1979; Kuwabara et al., 1991). For calculation of the concentration of OH radicals which actually react with oligomers in the presence of MNP, the other part of the solution containing MNP alone was used to determine the concentrations of the monomer and dimer of MNP after the same treatments. The concentration of the dimer was determined by UV absorbance (ϵ = 380 at 320 nm; unpublished results). Since the UV absorbance of monomer was quite low (ϵ = ca. 20), the monomer concentration was estimated by NMR measurements using *t*-BuOD as a standard. Finally, the N_2O-saturated aqueous solution containing the oligomer and MNP was exposed to X-rays to force the oligomer to react with OH radicals.

Treatment of OH-Radical-Attacked Oligomers with Piperidine. After X-ray irradiation of the solution without MNP, the solution in each tube was dried by centrifugal evaporation. One milliliter of 1 M piperidine was added to the dried sample, and the mixture was heated at 90 °C for 30 min. The solution was dried by centrifugal evaporation. The oligomer was redissolved in 200 μL of triply distilled water.

PAGE and Autoradiography of 5′-End-Labeled Oligo(dC)$_{10}$ and Oligo(dT)$_{10}$ after OH Radical Reactions. Analysis of OH-radical-attacked ^{32}P-oligomers was carried out on 19% polyacrylamide gels that contained 50% urea, essentially according to the method described by Samrook et al. (1989). After X-ray irradiation or X-ray irradiation plus piperidine treatment, each solution was dried and redissolved in 3 μL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll type 400). Polyacrylamide gels were run in the TBE buffer (89 mM Tris–borate, 89 mM boric acid, 1 mM EDTA, pH 8.0). After running, the gels were dried with a gel dryer at 50 °C for 3 h. The dried gels were layered on X-ray film (Kodak Diagnostic Film, Eastman Kodak, Rochester, NY) in a cassette. The films were exposed to β-rays from the gels for 12 h and developed. The dried gels were also analyzed with a Fujix 2000 Bio Image Analyzer (Fujix, Tokyo, Japan). The image plate was first exposed to the dried gels in the cassette and then inserted into the image reader. The luminescent-light intensities from the plate, which recorded bands corresponding to monomers to decamers, were measured and used for quantitative estimation of fragment formation.

RESULTS

ESR Spectra Obtained from Oligo(dC)$_{10}$ and Oligo(dT)$_{10}$. An N_2O-saturated aqueous solution was exposed to X-rays to generate OH radicals. Some OH radicals are consumed by reactions with the spin-trapping reagent itself to produce byproducts, but other OH radicals react with the solute (oligo(dC)$_{10}$ or oligo(dT)$_{10}$) to produce radicals which are subsequently trapped by the spin-trapping reagent (Kuwabara et al., 1989). When a nitroso compound such as MNP is used, the following spin-trapping reaction is expected to produce the spin adducts:

$$OH^{\cdot} + \text{oligonucleotide} \rightarrow \text{oligonucleotide radicals } (R^{\cdot}) \quad (1)$$

$$R^{\cdot} + t\text{-Bu—N=O} \rightarrow t\text{-Bu—N(O}^{\cdot})R \rightarrow t\text{-Bu—N}^{+}(O^{-})R \quad (2)$$

The ESR spectrum of the spin adduct has a primary triplet

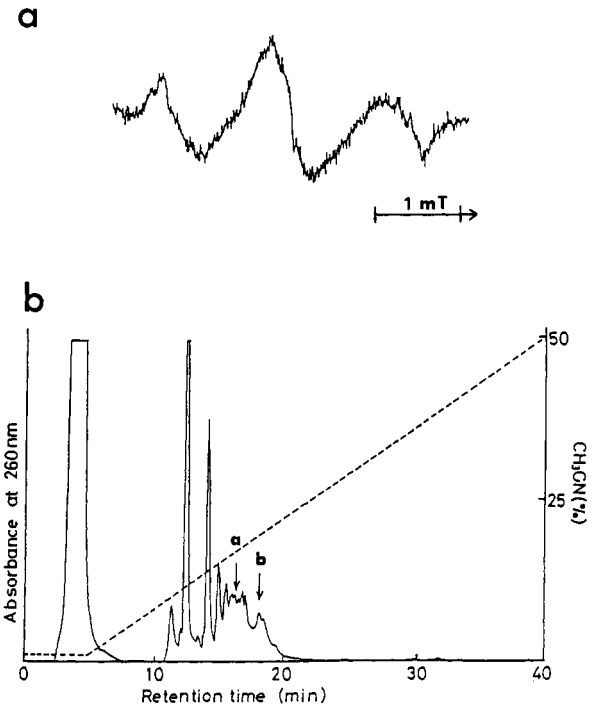


FIGURE 1: (a) ESR spectrum of spin-trapped radicals from an N_2O-saturated aqueous solution containing 2 mg/mL of oligo(dC)$_{10}$ and 1.3 mg/mL of MNP after irradiation with 2.7 kGy of X-rays. (b) Elution profile after 1 mL of N_2O-saturated aqueous solution containing oligo(dC)$_{10}$ and MNP was irradiated with X-rays and subsequently treated with 0.2 units of snake venom phosphodiesterase for 1 h. The fragments liberated from the oligomer were first separated by centrifugation using a centrifugal filtration tube and then separated by reverse-phase HPLC. Separation conditions are described in the text.

hyperfine structure due to the ^{14}N nucleus and a secondary hyperfine structure that arises from the magnetic nucleus (H or N) of the trapped radical R$^{\cdot}$. These primary and secondary hyperfine structures are utilized to identify the chemical structure of the R$^{\cdot}$. The ESR spectrum obtained from oligo(dC)$_{10}$ was broad and featureless due to slow tumbling of nitroxide spin adducts with a large molecular weight (Figure 1a). After digestion of the oligomer by phosphodiesterase, HPLC with a reverse-phase column was employed to separate and identify the spin adducts. A chromatogram was obtained by UV absorbance at 260 nm (Figure 1b). After undamaged dCMP molecules were eluted as a major constituent of the solutes, several peaks appeared in the chromatographic profile. When each fraction was examined by the ESR technique, only two signals were detected in fractions a and b. The ESR spectra obtained from the two peaks are shown in Figure 2. The spectrum of the spin adduct in fraction a showed a hyperfine structure consisting of a primary triplet of 1.53 mT and a secondary doublet of 0.37 mT, suggesting that a proton at the β-position in the spin adduct interacted with a spin of the nitroxide group. The spectrum of the spin adduct in fraction b showed a hyperfine structure consisting of only a primary triplet of 1.52 mT, suggesting that no protons or nitrogens were present at the β-position in the spin adduct. Our previous spin-trapping study of OH-radical-induced radicals in cytosine-related compounds led us to assign spectra a and b to the spin adduct between the C5-base radical and MNP and the spin adduct between the C4′-sugar radical and MNP, respectively (Hiraoka et al., 1990). Thus, the following radical species were assigned in OH-radical-exposed oligo(dC)$_{10}$.

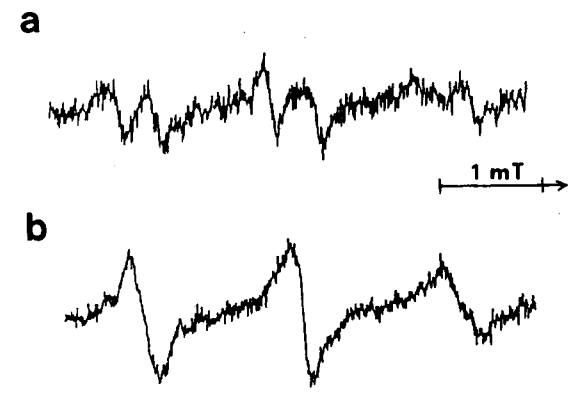
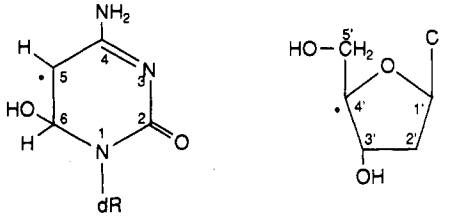


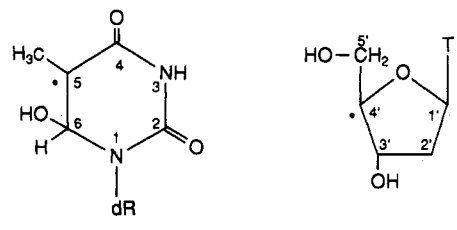
FIGURE 2: (a) ESR spectrum of the spin adduct contained in fraction a in Figure 1b. (b) ESR spectrum of the spin adduct contained in fraction b in Figure 1b.



C5 radical **C4' radical**

The C5-base radical is regarded as a precursor of cytosine base damage (Téoule, 1987), and the C4′-sugar radical is regarded as a precursor of DNA strand breaks (von Sonntag, 1980; Teebor et al., 1988).

A relatively sharp ESR spectrum was obtained from oligo-$(dT)_{10}$ (Figure 3a). After digestion of the oligomer by phosphodiesterase, an HPLC elution profile of the digested spin adducts of oligo$(dT)_{10}$ was obtained and is shown in Figure 3b. Examination of each fraction by ESR showed ESR signals in the fractions denoted a–c. The ESR spectrum of the spin adduct in fraction a showed a hyperfine structure consisting of a primary triplet of 1.60 mT without further splittings, indicating that this spin adduct had no protons or nitrogens at the β-position (Figure 4a). In the case of the ESR spectrum in fraction b (Figure 4b), though the main ESR spectrum was largely contaminated by the spin adduct belonging to fraction c (Figure 4c), we could analyze the ESR spectrum as having a primary triplet of 1.48 mT and a secondary triplet of about 0.28 mT, indicating that the spin adduct had a nitrogen at the β-position. The ESR spectrum in Figure 4c consisted of a primary triplet of 1.58 mT without further splittings. In a previous spin-trapping study of TMP, three ESR spectra, which were similar to those in Figures 4a and 4c, and two other ESR spectra, which were similar to that in Figure 4b, were obtained (Kuwabara et al., 1989). Thus, two stereoisomers of the spin adducts at the C5 position of the base moiety and the spin adduct at the C4′ position of the sugar moiety gave ESR spectra similar to each other, as each had a primary triplet alone:



***cis* & *trans* C5 radicals** **C4' radical**

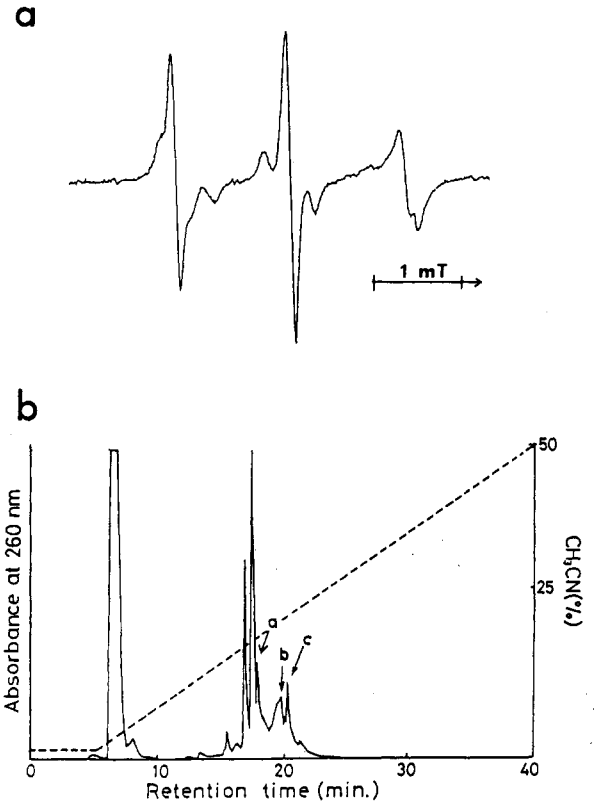
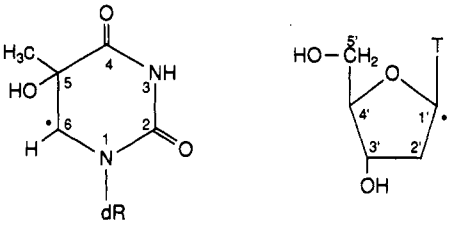


FIGURE 3: (a) ESR spectrum of spin-trapped radicals from an N_2O-saturated aqueous solution containing 2 mg/mL of oligo$(dT)_{10}$ and 1.3 mg/mL of MNP after irradiation with 2.7 kGy of X-rays. (b) Elution profile after 1 mL of N_2O-saturated aqueous solution containing oligo$(dT)_{10}$ and MNP was irradiated with X-rays and subsequently treated with 0.2 units of snake venom phosphodiesterase for 1 h. The fragments liberated from the oligomer were first separated by centrifugation using a centrifugal filtration tube and then separated by reverse-phase HPLC. Separation conditions are described in the text.

Similarly, the spin adduct at the C6 position of the base moiety and the spin adduct at the C1′ position of the sugar moiety gave ESR spectra similar to each other, as each had a primary triplet with a secondary triplet:



C6 radical **C1' radical**

Thus, three candidates are present for Figures 4a and 4c, and two candidates are present for Figure 4b. From the hyperfine-splitting constants and the order of HPLC separation, Figures 4a, 4b and 4c were assigned to the C4′-sugar radical, the C6-base radical, and the C5-base radical, respectively. Assignment of Figure 4b to the C6-base radical rather than the C1′-sugar radical was plausible, because OH radicals more frequently attacked the base moiety than the sugar moiety in oligo$(dT)_{10}$ (see below). However, this fact also seemed to support the attribution of both Figures 4a and 4c to the stereoisomers of the C5-base radicals. Therefore, evidence for the formation of sugar radicals as strand-break precursors had to be obtained by another method. For this purpose, each oligomer was ^{32}P-labeled at the C5′ terminus and exposed to

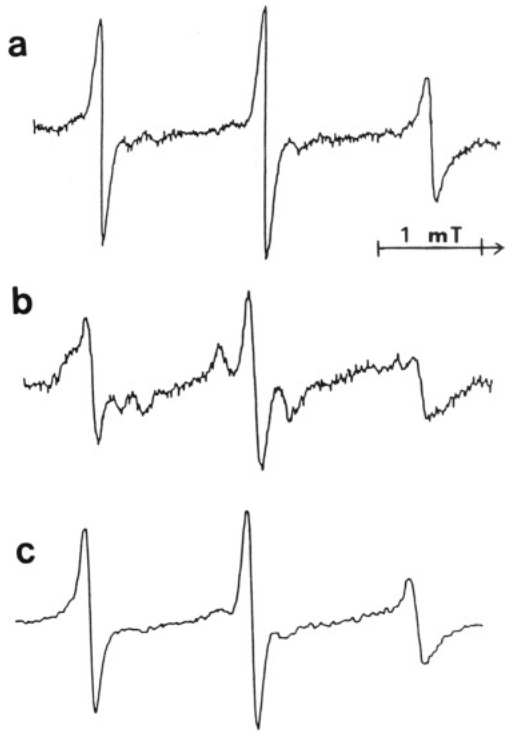


FIGURE 4: ESR spectra obtained from fractions a, b, and c in Figure 3b. Spectra a, b, and c correspond to fractions a, b, and c, respectively.

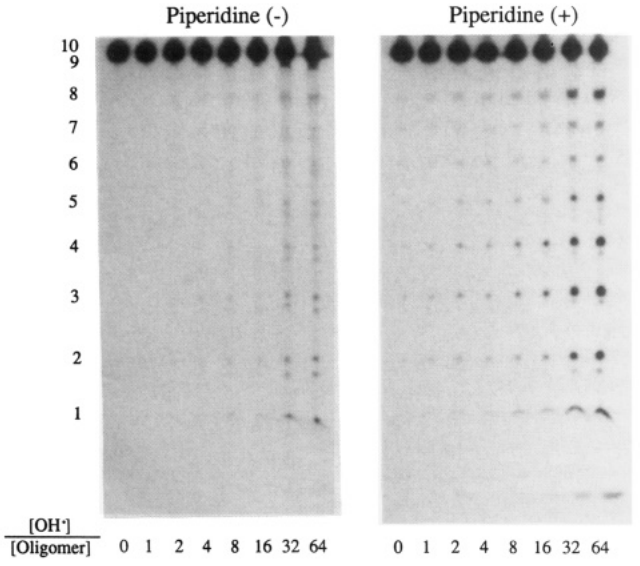


FIGURE 5: PAGE patterns of 5′-^{32}P-end-labeled oligo(dC)$_{10}$ after OH radical attack. 5′-^{32}P-end-labeled oligo(dC)$_{10}$ was mixed with nonlabeled oligo(dC)$_{10}$ to become OD_{260nm} = 0.31. After the solution containing the mixture was saturated with N_2O gas, it was exposed to X-rays. The molar ratio of the OH radical concentration to the oligomer concentration is shown at the bottom of the autoradiogram. The autoradiograms of 5′-^{32}P-end-labeled oligo(dC)$_{10}$ after treatment with X-rays alone and with X-rays and piperidine are shown in the left and right lanes, respectively.

OH radicals in the presence or in the absence of MNP. PAGE of each oligomer was carried out before and after OH-radical reactions.

PAGE Analysis of 5′-End-Labeled Oligo(dC)$_{10}$ and Oligo(dT)$_{10}$ Treated with OH Radicals in the Absence of MNP. Figure 5 demonstrates that increases in the concentrations of OH radicals led to an increase in the extent of strand breaks in oligo(dC)$_{10}$, as evidenced by the increase of the luminescence intensities that migrated more rapidly than those of full-sized oligomers (left). Fragments corresponding to the monomer

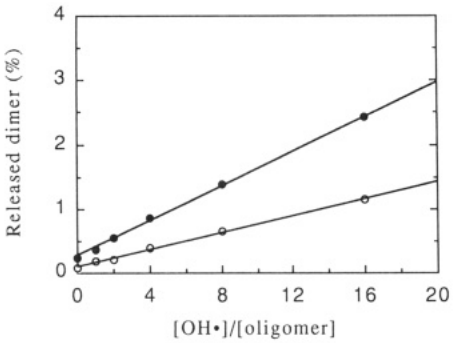


FIGURE 6: Plots of the released dimer vs [OH·]/[oligo(dC)$_{10}$], where the released dimer (%) was obtained by dividing the sum of luminescence intensities of two spots of the corresponding dimer by the sum of those of all spots. [OH·]/[oligo(dC)$_{10}$] is the ratio of the molar concentration of OH radicals which were generated by water radiolysis and the molar concentration of the oligomer. The quantitative data were obtained by analyzing the gel corresponding to Figure 5 with a Fujix 2000 Bio Image Analyzer as described in the text.

to the 9mer sequence appeared. Each fragment consisted of a band with a pair of spots. When oligo(dC)$_{10}$ was treated with piperidine after irradiation, the intensity of the upper band of the pair rather than that of the lower one changed (right). Since piperidine treatment of OH-radical-attacked DNA can be used to visualize the OH-radical-induced modification of the base moiety to strand breaks with a phosphate group at the 3′ terminus (Maxam & Gilbert, 1980), the upper band was regarded as a fragment with a simple phosphate group at the 3′ terminus. As for the lower band, since Henner et al. (1982, 1983) reported that γ-ray irradiation of DNA produced strand breaks with two types of 3′ termini, a simple phosphoryl group and an altered sugar (glycolic acid) attached to the 3′-phosphoryl group, the lower spot was regarded as a fragment having a similar chemical structure at the 3′ terminus. However, Beesk et al. (1979) reported another type of 3′-phosphate-altered sugar (2′,3′-dideoxy-pentos-4-ulose). These researchers exposed DNA to γ-rays under different conditions (Henner et al. irradiated in oxygenated solution and Beesk et al. irradiated in anoxic solution). Though the anoxic conditions were employed in the present experiment, we examined oligomers with PAGE to determine whether such analysis could distinguish between two chemical structures. When the oligomers were exposed to X-rays under anoxic and oxygenated conditions and subsequently analyzed by PAGE, similar PAGE patterns were obtained under both types of conditions (data not shown). This meant that no conclusive evidence for the chemical structure of altered sugar could be induced from the present experiment.

When the luminescence intensities corresponding to the released dimer (the sum of luminescence intensities of both bands) were plotted against [OH·]/[oligomer] before and after piperidine treatment, straight lines were obtained, and the slope (i.e., the efficiency of the dimer release from the oligomer) became almost twice as steep after piperidine treatment (Figure 6). This meant that the amount of base damage was almost equal to that of sugar damage; in other words, that OH radicals reacted with the base and sugar moieties with almost equal probability in the case of oligo(dC)$_{10}$. Similar results were observed in other fragments.

Figure 7 demonstrates the strand breaks of oligo(dT)$_{10}$ induced by OH radicals (left). In a manner similar to that of oligo(dC)$_{10}$, fragments corresponding to the monomer to the 9mer sequence appeared, and the intensity of the upper

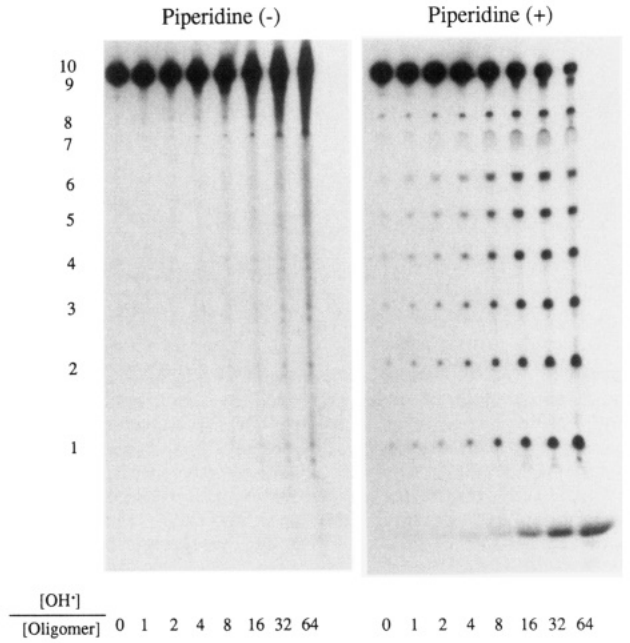


FIGURE 7: PAGE patterns of 5′-^{32}P-end-labeled oligo(dT)$_{10}$ after OH radical attack. 5′-^{32}P-end-labeled oligo(dT)$_{10}$ was mixed with nonlabeled oligo(dT)$_{10}$ to become OD_{260nm} = 0.3. After the solution containing the mixture was saturated with N_2O gas, it was exposed to X-rays. The molar ratio of the OH radical concentration to the oligomer concentration is shown at the bottom of the autoradiogram. The autoradiograms of 5′-^{32}P-end-labeled oligo(dT)$_{10}$ after treatment with X-rays alone and with X-rays and piperidine are shown in the left and right lanes, respectively.

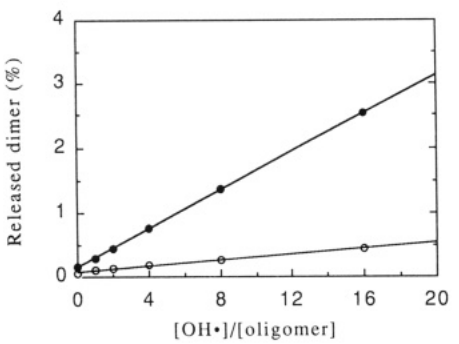


FIGURE 8: Plots of the released dimer vs [OH·]/[oligo(dT)$_{10}$], where the released dimer (%) was obtained by dividing the sum of luminescence intensities of two spots of the corresponding dimer by the sum of those of all spots. [OH·]/[oligo(dT)$_{10}$] is the ratio of the molar concentration of OH radicals which were generated by water radiolysis and the molar concentration of the oligomer. The quantitative data were obtained by analyzing the gel corresponding to Figure 7 with a Fujix 2000 Bio Image Analyzer as described in the text.

band of a pair of spots became stronger after the piperidine treatment (right). These results led us to the same conclusion as for oligo(dC)$_{10}$; the upper band was regarded as a fragment with a simple phosphate group at the 3′ terminus. When the luminescence intensities (the sum of luminescence intensities of both bands) corresponding to the released dimer were plotted against [OH·]/[oligomer] before and after piperidine treatment, straight lines were again obtained (Figure 8), but the slope (i.e., the efficiency of dimer release from the oligomer) was about one-third of that of oligo(dC)$_{10}$. The piperidine-treated oligo(dT)$_{10}$ showed a line slope quite similar to that of oligo(dC)$_{10}$. This meant that the total amounts of damage were almost equal in both oligomers, and, in other words, that OH radicals reacted with the base moiety in preference to the sugar moiety in the case of oligo(dT)$_{10}$.

PAGE Analysis of 5-End-Labeled Oligo(dC)$_{10}$ and Oligo(dT)$_{10}$ Treated with OH Radicals in the Presence of MNP. As described in Methods, aqueous solutions containing 0.2 mg of MNP and 4.1 μM oligomer (OD_{260nm} = 0.31 and 0.39 for oligo(dC)$_{10}$ and oligo(dT)$_{10}$, respectively) were prepared. In spin-trapping experiments, a relatively high concentration of MNP (1.3 mg/mL) was used. This was empirically chosen because the monomer form of MNP which actually served as a spin-trapping reagent was volatile, and its concentration sometimes fell for various reasons (Riesz & Rustgi, 1979). In this case, a low concentration of MNP (the same order of concentration as those of oligomers) was chosen because this compound reacted with OH radicals in a competitive fashion with oligomers. After the concentrations of the monomer and dimer of MNP were carefully measured, the reaction rate constants between OH radicals and the monomer and dimer of MNP were determined to be 2×10^{10} and 2.8×10^{9} M^{-1} s^{-1}, respectively, by the pulse radiolysis method (unpublished results). The protective effects of MNP against fragmentation were then calculated by introducing simple scavenging of OH radicals with the monomer and dimer of MNP. The results are shown in Figure 9 in addition to the experimentally observed ones. The slopes of the experimentally obtained lines (●) were found to be gentler than those of the theoretically calculated ones (*) in both oligomers. These results suggest that MNP protected against OH-radical-induced fragmentation of oligomers not only by simple OH scavenging but also in another way. To explain the additional protective effect of MNP, one is forced to accept that the fragmentation was suppressed by the trapping of strand-break precursor radicals by the MNP monomer. Consequently, the ESR spectra in Figure 2a and in either Figure 4a or 4c can be assigned to the C4′-sugar radical, which is regarded as a strand-break precursor. As for Figures 4a and 4c in oligo(dT)$_{10}$, taking into account the hyperfine-splitting constants and the order of HPLC separation, Figures 4a and 4c were preferred for the assignment of the C4′ radical and the C5-base radical, respectively (Kuwabara et al., 1989). In this experiment, it was not easy to measure exactly the concentration of the monomer, and, therefore, determination of the reaction rate constant between the OH radical and the monomer contained an uncertainty. The rate constant (2×10^{10} M^{-1} s^{-1}) used in this calculation seemed to be relatively high, but this value was chosen to avoid underestimation of the OH-scavenging effect due to the MNP monomer.

DISCUSSION

The spin-trapping experiments with both oligonucleotides identified only one radical species, i.e., the C4′ radical at the sugar moiety, as a candidate for the precursor of strand breaks. Therefore, the formation of the two 3′ termini must be explained by the C4′ radical. In fact, Beesk et al. (1979) and Henner et al. (1982, 1983) reported that γ-ray irradiation of DNA produced strand breaks with two types of 3′ termini and presumed that the C4′ radical was a precursor of these termini. They reported different chemical structures for the 3′ termini. Beesk et al. (1979) reported 3′-phosphate and 3′-phosphate-altered sugar (2′,3′-dideoxypentos-4-ulose), and Henner et al. (1982, 1983) and Schulte-Frohlinde and Bothe (1984) identified 3′-phosphate and 3′-phosphate-altered sugar (glycolic acid). The former carried out γ-ray irradiation in the absence of oxygen, and the latter carried out γ-ray irradiation in the presence of oxygen. Giloni et al. (1981) reported that bleomycin-induced DNA strand scission consisted of 3′-phosphoglycolic acid and required the addition of molecular oxygen to the C4′ radical for its formation. When the

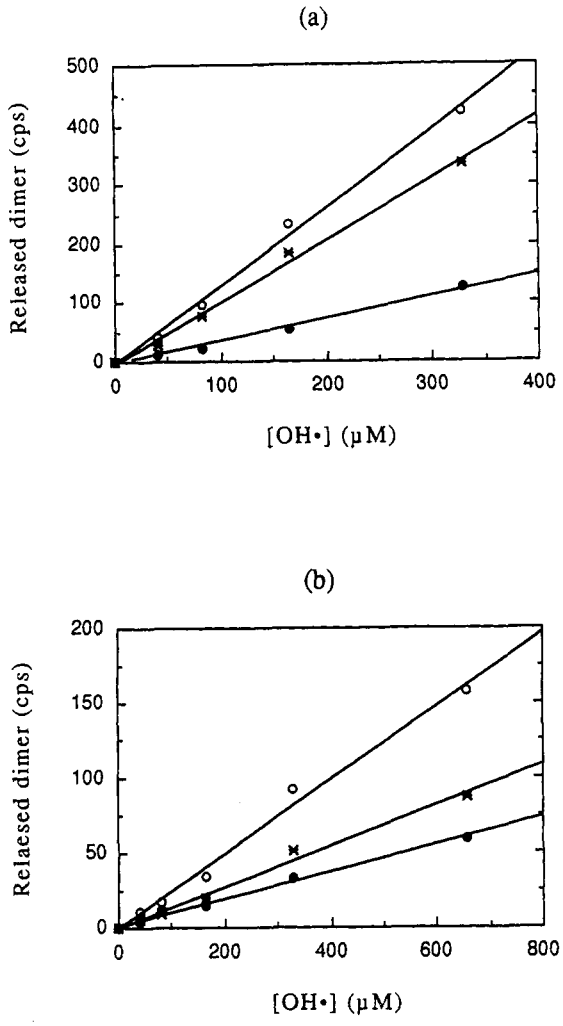


FIGURE 9: Plots of the released dimer vs [OH·], where the released dimer was expressed as counts of luminescence intensities of spots. The quantitative data were obtained by analyzing the gels for (a) oligo(dC)$_{10}$ and (b) oligo(dT)$_{10}$ with a Fujix 2000 Bio Image Analyzer. (○) Data obtained after X-ray irradiation in the absence of MNP, (✱) theoretical data calculated according to the competitive reaction model between OH radicals and the monomer and dimer of MNP using the rate constants 2×10^{10} M^{-1} s^{-1} for the monomer and 2.8×10^{9} M^{-1} s^{-1} for the dimer (unpublished results), and (●) data experimentally obtained after X-ray irradiation in the presence of MNP.

oligomers were exposed to X-rays under both anoxic and oxygenated conditions and subsequently analyzed by PAGE, no difference in the PAGE patterns was observed between the two conditions. Therefore, the present experiments could not distinguish between the chemical structure of the OH-radical-induced 3′ terminus under anoxic conditions and that produced under oxygenated conditions.

Oligo(dC)$_{10}$ has two OH-radical-reactive sites (C5, C6 double bond) at the base moiety and five OH-radical-reactive sites (C1′ to C5′) at the sugar moiety, while oligo(dT)$_{10}$ has three OH-radical-reactive sites (C5, C6 double bond, and C5-methyl group) at the base moiety and five OH-radical-reactive sites (C1′ to C5′) at the sugar moiety. However, only one base radical (C5 radical) and one sugar radical (C4′ radical) were identified in oligo(dC)$_{10}$, and two base radicals (C5 and C6 radical) and one sugar radical (C4′ radical) were identified in oligo(dT)$_{10}$. These results were in contrast to those obtained from their constituents (thymine- and cytosine-related compounds), in which the C5, C6, and C5 methyl radicals at the base moiety and the C1′, C4′, and C5′ radicals at the sugar moiety were identified (Kuwabara et al., 1987, 1989; Hiraoka et al., 1990). The difference between them may be interpreted as suggesting that the polymerized forms of mononucleotides minimize the sites at which OH radicals can attack or restrict the sites at which MNP can trap spins. The susceptibility of bases in DNA to attack by OH radicals generated through water radiolysis seems to be less than that of free bases [see, for example, the review by von Sonntag (1987)].

The spin-trapping experiments with oligo(dC)$_{10}$ and oligo(dT)$_{10}$ gave ESR spectra which were attributable to the C4′ radical at the sugar moiety, suggesting it as a candidate for the precursor of strand breaks (Figures 2b and 4a). Examination of OH-radical-attacked oligomers in the presence or absence of MNP with PAGE supported the conclusion that the assignments of these two ESR spectra, especially the ESR spectrum obtained from oligo(dT)$_{10}$, to the C4′ radical were reasonable. Interestingly, the gel electrophoresis patterns clearly showed that reactivities of OH radicals with the base and sugar moieties were different in oligo(dC)$_{10}$ and oligo(dT)$_{10}$. When OH radicals encounter a cluster of dCMP in DNA, half of them may react with the sugar moiety to induce DNA strand scission, but when OH radicals encounter a cluster of TMP, most OH radicals may react with the thymine base moiety, indicating that the thymine base may play a role in protecting DNA from strand breaks due to OH radicals. Since the presence of thymine-rich and cytosine-rich regions in actual DNA has been reported, it is possible that OH radicals do not react uniformly with DNA to induce strand breaks. In a previous paper we reported that when OH-radical-induced free radicals in calf thymus DNA were trapped by MNP, the majority of the components of the observed ESR spectrum was due to the spin adducts between thymine base radicals and MNP (Kuwabara et al., 1987). This might be a reflection of OH-radical reactivities in the thymine-rich region in DNA. From the present study it was concluded that a method combining spin trapping and gel electrophoresis was a good approach to identify sites of radical damage which cause strand breaks in macromolecules such as DNA.

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