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Spin Trapping of Precursors of Thymine Damage in X-Irradiated DNA†

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ABSTRACT: A spin-trapping method combined with ESR spectroscopy was utilized to obtain evidence for the presence of precursor radicals leading to damage in X-irradiated DNA. Two technical improvements were introduced to the conventional spin-trapping method to make possible its application to large molecules such as DNA: (1) prior to X irradiation, sonolysis of aqueous DNA solution by 19.5-kHz ultrasound was made to get a highly concentrated DNA solution and to lower the viscosity of the solution; (2) after precursor radicals in X-irradiated DNA were trapped by a spin-trapping reagent, the DNA was digested to oligonucleotides by DNase I to get an ESR spectrum with a well-resolved hyperfine structure. Thus, it was recognized that the ESR spectrum obtained after X irradiation of the aqueous solution containing DNA and the nitroso spin-trapping reagent 2-methyl-2-nitrosopropane consisted of at least three sets of signals in the DNA. Identification of free radicals was made by comparing the spectrum with that of thymidine, which was precisely examined by a spin-trapping method combining two kinds of spin traps (nitroso and nitrone compounds) with liquid chromatography. As a result, all the signals were identified as the spin adducts of radicals produced at the thymine base moiety of DNA. The 5-hydroxy-5,6-dihydrothymin-6-yl radical was identified as a precursor of 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol), the 6-hydroxy-5,6-dihydrothymin-5-yl radical as a precursor of 6-hydroxy-5,6-dihydrothymine, and the 5-methyleneuracil radical as a precursor of 5-(hydroxymethyl)uracil.

OH radicals are generated via arachidonic acid metabolism in platelets, leukocytes, macrophages, hemangioendothelial cells, and microsomes, as well as in electron transport systems in mitochondria. They are regarded as potential inducers of cellular activity and tissue responses such as inflammation (Kuehl & Egan, 1980; Murota, 1984), whereas ionizing radiation is well-known to be the most convenient tool for gen-

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erating OH radicals from water within a cell and allowing them to react randomly with each component of the cell.

Many radiobiological studies have given evidence that the reactions of OH radicals with DNA in all cellular components lead to the most serious effects (Chapman & Gillespie, 1981). OH radicals cause two typical reactions in DNA to give rise to unstable and reactive intermediates (free radicals): (1) hydrogen abstraction from the saturated deoxyribose moiety; (2) addition reaction to unsaturated double bonds at the base moiety.

$$OH^{\bullet} + RH \rightarrow R^{\bullet} + H_2O \quad (1)$$

$$OH^{\bullet} + RH \rightarrow RHOH^{\bullet} \quad (2)$$

Here RH denotes the DNA molecule. These intermediates undergo a variety of radical decompositions and cause various kinds of DNA damage, such as base destruction, strand breaks, and alkaline-labile lesions.

$$R^{\bullet}\ (RHOH^{\bullet}) \rightarrow \text{DNA damage} \quad (3)$$

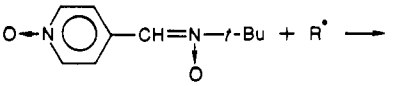
The chemical structures of OH-induced base damage have been reported by several investigators in cases of both isolated and cellular DNA (Hariharan & Cerutti, 1972; Têoule & Cadet, 1978; Frenkel et al., 1981a,b, 1985; Teebor et al., 1982, 1984; Leadon & Hanawalt, 1983; Breimer & Lindahl, 1985). In these studies, the thymine base was particulary susceptible to OH attack, and some oxidative conversions were found to cause major damage in DNA. For the end group of strand breaks, many structures have been proposed by using isolated DNA, but only a few structures have been elucidated at the cellular level (von Sonntag & Schulte-Frohlinde, 1978; von Sonntag, 1980; Hagen et al., 1980; von Sonntag et al., 1981; Henner et al., 1982, 1983a,b; Schulte-Frohlinde & Bothe, 1984).

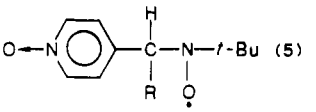
In this study we have employed a spin-trapping method to detect and identify OH-induced intermediates of DNA that lead to critical lesions in living cells. The investigation of radical intermediates leading to such DNA damage has generally been carried out with bases, nucleosides, and nucleotides. It is generally known that studies of organic free radicals by the ESR[1] method frequently face some difficulty because of the shortness of their lifetimes. The technique of spin trapping has been used to make it easier to measure radicals with a conventional ESR spectrometer by converting short-lived radicals ($R^{\bullet}$) to long-lived nitroxide radicals (the spin adduct) (Janzen, 1971; Lagercranz, 1971). If a nitroso compound such as MNP is used, the spin adducts are formed by the reaction:

$$R^{\bullet} + t\text{-Bu—N=O} \rightarrow t\text{-Bu—N(O}^{\bullet})R \rightarrow t\text{-Bu—}\dot{N}^{+}(O^{-})R \quad (4)$$

The ESR spectrum of the spin-adduct nitroxide shows a primary triplet splitting due to the ^{14}N nucleus and secondary splittings that arise from the magnetic nuclei of the trapped radical $R^{\bullet}$. These primary and secondary splittings are utilized to identify the chemical structure of the $R^{\bullet}$.

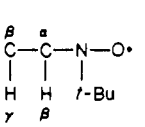
Other spin traps frequently employed are nitrone compounds such as 4-PyOBN, developed by Janzen et al. (1978). In this case the spin adducts are formed by the reaction:

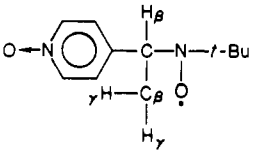




(5)

The secondary structure of the ESR spectrum shows a proton doublet without further splittings or a proton doublet accompanied by hfs due to another magnetic nucleus. This is useful in determining, for example, whether a carbon-centered or a nitrogen-centered radical, such as $R^{\bullet}$, is trapped (Spalletta & Bernhard, 1982). The α-, β-, and γ-positions of the magnetic nuclei are defined with respect to the unpaired electron on the nitrogen atom of the nitroxide group as illustrated:





Good results have already been reported by several authors who applied this method to nucleic acid constituents (Joshi et al., 1976; Kominami et al., 1976, 1977; Riesz & Rustgi, 1979; Kuwabara et al., 1981b, 1982, 1986; Inanami et al., 1986). In the present study, we first carried out the spin trapping of dThd, a constituent of DNA, by the use of both nitroso (MNP) and nitrone (4-PyOBN) compounds in combination with liquid chromatography. This was done in order to present unambiguous evidence for the assignments of OH-induced intermediates in X-irradiated DNA. The spin trapping of DNA was performed subsequently.

There have been some limitations in applying this method to macromolecules such as DNA and proteins, because of their low solubility in aqueous solution and the less efficient trapping of radicals due to the high viscosity of the solution. Furthermore, the ESR spectrum of the spin adduct has a broader line width and a poorly resolved hyperfine structure, due to the slow tumbling of the macromolecules (Joshi et al., 1976). To overcome these difficulties, we have introduced two technical improvements to the conventional spin-trapping method. First, prior to X irradiation, aqueous DNA solution is exposed to 19.5-kHz ultrasound to obtain a highly concentrated DNA solution and to lower the viscosity of the solution, thereby increasing the trapping efficiency of radicals by spin-trapping reagents. Second, the adducts of DNA and the spin-trapping reagent were digested to oligonucleotides by DNase I to generate an ESR spectrum with a well-resolved hyperfine structure. We will first present the results obtained from the spin trapping of dThd and then discuss the results of the examination of DNA on the basis of the dThd experiments.

Experimental Procedures

Materials. According to the method of Kay et al. (1952), DNA was extracted from calf thymus. dThd, bovine pancreas DNase I (type I, EC 3.1.4.5), and RNase A (type X-A, EC 3.1.27.5) were purchased from Sigma. Yeast RNA was purchased from Wako Pure Chemicals. This was purified once by a Sephadex G-25 column before use. MNP and 4-PyOBN were acquired from Aldrich.

OH Generation. OH radicals were generated by X irradiation of water. When an N_2O-saturated aqueous solution is exposed to 1 Gy (1 J/kg) of X-rays, about 0.54 μmol of OH radical is generated because hydrated electrons (e_{aq}^{-}) are converted to OH radicals by the reaction:

[1] Abbreviations: ESR, electron spin resonance; MNP, 2-methyl-2-nitrosopropane; 4-PyOBN, α-(1-oxy-4-pyridyl)-*N*-*tert*-butylnitrone; DNase I, deoxyribonuclease I; RNase A, ribonuclease A; dThd, thymidine; TMP, thymidine 5′-monophosphate; hfs, hyperfine splitting; *t*-Bu, *tert*-butyl.

$$N_2O + e_{aq}^- + H_2O \rightarrow OH^\bullet + OH^- + N_2 \quad (6)$$

In the case of water containing air, about 0.27 μmol of OH radicals is generated and e_{aq}^- are effectively scavenged by O_2 to form O_2^-. X irradiation was carried out by operating a Toshiba X-ray machine at 180 kV and 25 mA.

Spin Trapping of OH-Induced Radicals in dThd by MNP and 4-PyOBN and Liquid Chromatography. MNP (1 mg) was put into 1 mL of H_2O solution containing 20 mM dThd (this corresponds to 10 mM MNP). The powder was then dissolved by stirring the solution overnight at 25 °C in the dark to get MNP monomer–dimer equilibrium. In the experiment using 4-PyOBN, 2 mM 4-PyOBN and 20 mM dThd were simultaneously dissolved in 1 mL of H_2O. Both solutions were exposed to X-rays at a dose of 2.7 kGy. The resulting spin adducts were chromatographed on a Sep-Pak C_{18} cartridge (Waters Associates, Inc.) with a methanol–water mixture as the eluent. The concentration of methanol was increased from 0% to 60% by steps of 10%. Each of the seven fractions was examined by an ESR spectrometer at room temperature using an aqueous flat cell (60 × 10 × 0.25 mm). The ESR measurements were made on a JEOL ME X-band spectrometer. The ESR spectra from the spin-trapped radicals were recorded as the first derivative. The majority of the ESR scans were traced with 100-kHz modulation of 0.02-mT amplitude and 20-mW microwave power.

Spin Trapping of Hydrated Electron Induced Radicals in dThd by MNP and 4-PyOBN. MNP powder (1 mg) was added to 1 mL of H_2O solution containing 20 mM dThd and 1 M sodium formate. The solution was saturated with Ar gas by bubbling for 20 min. The powder was then dissolved by stirring the solution overnight at 25 °C. Since the monomer, which is the effective form for spin trapping, is readily removed by bubbling, this process must be carried out before dissolving the MNP powder. In the experiment using 4-PyOBN, 20 mM dThd, 1 M sodium formate, and 2 mM 4-PyOBN were simultaneously dissolved in 1 mL of H_2O. Saturation of the solution by Ar gas was carried out after they were dissolved, since the bubbling brought about no elimination of 4-PyOBN. These solutions were exposed to X-rays at a dose of 4 kGy at 77 K to avoid the formation of side products by the spin-trapping reagent itself (Kuwabara et al., 1983). After the solution was thawed at 45 °C, the ESR spectrum was recorded at room temperature.

Spin Trapping of Radicals in γ-Irradiated Solid dThd by MNP and 4-PyOBN. Spin trapping of radical intermediates formed in solid dThd by γ radiolysis was done essentially according to the method previously described (Kuwabara et al., 1981a). Polycrystalline dThd was irradiated in a ^{60}Co γ source at a dose of 50 kGy in an Ar atmosphere. By use of a glass U-tube, which was specially designed for spin-trapping studies in the absence of oxygen (Evans, 1979), the irradiated polycrystalline sample was placed in one side of the tube and 2 mg of MNP or 40 mg of 4-PyOBN and 1 mL of H_2O were placed in the other side of the tube. The MNP was dissolved by warming H_2O to 45 °C while stirring for 30 min after flushing Ar gas into the H_2O for 20 min through a fine needle inserted into a rubber septum. The 4-PyOBN was dissolved once in H_2O at room temperature, and then the oxygen was removed by Ar bubbling in the same manner as above. The H_2O solution containing the spin-trapping reagent was transferred to the side of the U-tube containing the preirradiated polycrystalline sample, followed by dissolving it by rapid stirring to induce spin-trapping reactions. Immediately after resolution, the solution was transferred to an ESR flat cell. The ESR measurements were made at room temperature. It should be noted that sodium formate was added to the solution at a concentration of 1 M in order to record the spectra under the same conditions as the spin trapping of hydrated electron induced radicals.

Spin Trapping of OH-Induced Radicals in DNA by MNP. Twenty milligrams of calf thymus DNA, which had an average molecular weight of 5.1 × 10^6, as determined by analytical ultracentrifugation (31 820 rpm, Hitachi UCA-1A), was put into 1 mL of triply distilled water. The DNA swelled and the solution became viscous. When the solution was exposed to 19.5-kHz ultrasound at 140 W for 20 min and dialyzed against distilled water overnight, it lost its viscosity and turned into a mobile liquid. The concentration of DNA was about 14 mg/mL, and the molecular weight decreased to 1.1 × 10^5. But since the DNA still maintained its molecular weight at a high level and no other serious damage was induced, it could be employed for the subsequent spin-trapping experiments. After 0.8 mg of MNP powder was added to a 1-mL DNA solution, the solution was saturated with N_2O gas, rather than air, by bubbling for 20 min. The solution was then stirred overnight in the dark at 25 °C to dissolve the powder. X irradiation was carried out at a dose of 2.7 kGy, corresponding to the generation of about 1.5 mM OH radicals. While part of the irradiated solution was immediately analyzed by ESR spectrometer at room temperature, the remainder was digested once by DNase I (100 μg/mL) in buffer solution (10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.8) at 37 °C for 30 min and subsequently analyzed by ESR spectrometer at 40 °C.

Results

ESR Spectra of Spin Adducts of dThd and MNP. In previous papers we have shown that certain types of radicals are difficult to trap by MNP in the presence of O_2, but not in the absence of O_2 (Kuwabara et al., 1981a; Zhang et al., 1983). This fact was utilized to minimize the number of spin adducts and to make it easier to analyze the composite spectrum. Figure 1A shows an ESR spectrum obtained when an aqueous solution containing dThd and MNP was X-irradiated in the presence of O_2. Figure 1B is an ESR spectrum obtained from a spin adduct present in a 10% methanol fraction when the spin adducts were separated by a Sep-Pak C_{18} cartridge with a methanol–water mixture. Figure 1C shows an ESR spectrum that appeared in a fraction eluted by 50% methanol solution. No signals other than these two were found.

Spectrum B (Figure 1) consists of a primary triplet of 1.44 mT which further splits into a secondary 1:1:1 triplet of 0.28 mT. The secondary triplet can be explained by the interaction of a spin with a nitrogen atom at the α- or β-position. Therefore, different assignments were made by several investigators. Joshi et al. (1976) assigned this spectrum to spin trapping at N3 of the base moiety. Kominami et al. (1977) assigned it to N1 or N3, whereas Kuwabara et al. (1983) assigned it to N3 or C6. The assignment of this spectrum to spin trapping at the C6 position was difficult since no hfs due to a β-proton present at C6 was included. However, analytical studies of end products in γ-irradiated thymidine clearly indicated that the products were formed via a C6-centered radical (Tēoule & Cadet, 1978). In the present paper, we carried out spin-trapping experiments using another kind of trapping reagent, 4-PyOBN, in order to obtain conclusive evidence concerning this discrepancy. As described above, direct addition of this trap to a nitrogen atom yields a β-nitrogen hfs, whereas addition to a carbon atom with an adjacent nitrogen atom will not yield a measurable secondary nitrogen hfs.

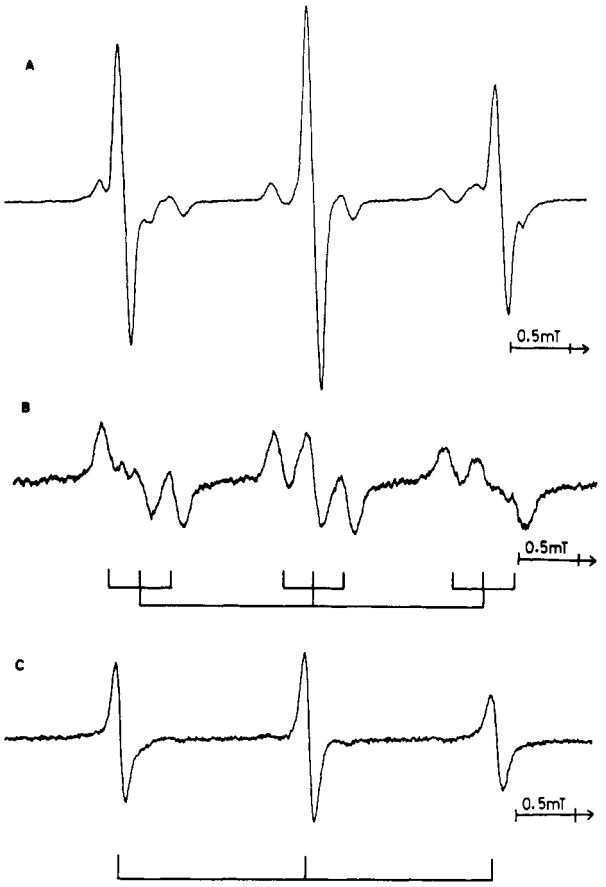


FIGURE 1: (A) ESR spectrum obtained after X irradiation (2.7 kGy) of an air-saturated aqueous solution containing dThd (20 mM) and MNP (10 mM), (B) ESR spectrum of a spin adduct recovered in a 10% methanol fraction when spin adducts were separated by a Sep-Pak C_{18} cartridge with a methanol–water mixture, and (C) ESR spectrum obtained from a spin adduct in a 50% methanol fraction.

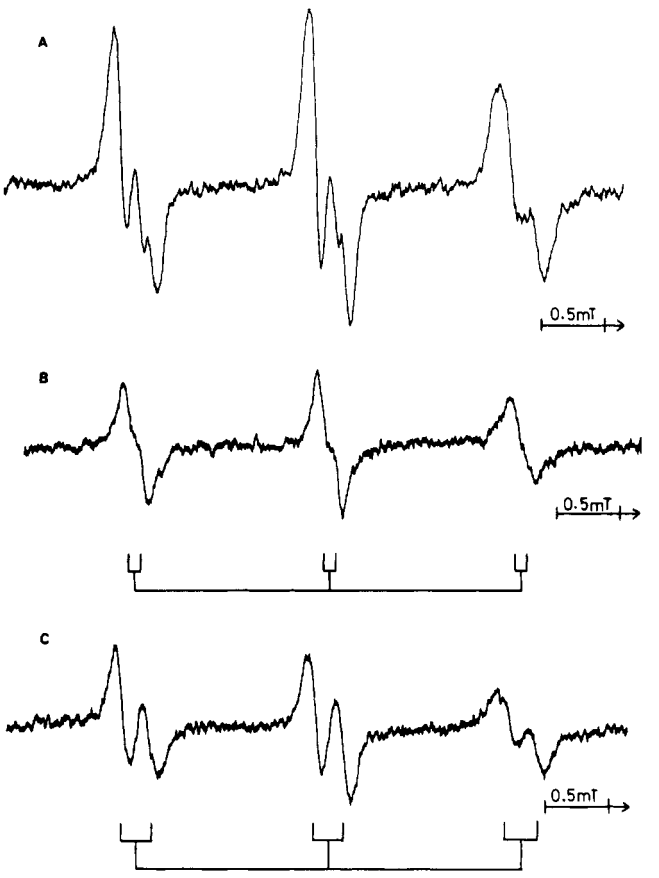
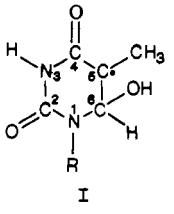


FIGURE 2: (A) ESR spectrum obtained after X irradiation (2.7 kGy) of an air-saturated aqueous solution containing dThd (20 mM) and 4-PyOBN (2 mM), (B) ESR spectrum of a spin adduct recovered in a 10% methanol fraction when spin adducts were separated by a Sep-Pak C_{18} cartridge with a methanol–water mixture, and (C) ESR spectrum of a spin adduct recovered in the 50% fraction.

Before describing the results obtained with 4-PyOBN, it will be useful to know the assignment of the spectrum shown in Figure 1C. Figure 1C consists only of a primary triplet. In other words, this means that there are no magnetic nuclei at the β-position in the spin-trapped form. This can be assigned to the spin-trapped radical at the C5 position induced by OH addition to the C6 position of the base moiety 6-hydroxy-5,6-dihydrothymin-5-yl radical (structure I). Similar spectra

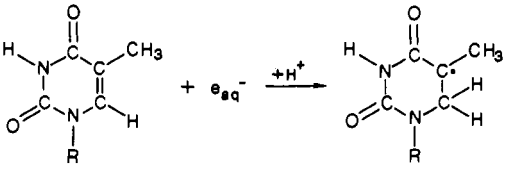


I

have already been reported for dThd and TMP (Joshi et al., 1976; Kominami et al., 1977; Kuwabara et al., 1982).

ESR Spectra of Spin Adducts of dThd and 4-PyOBN. The ESR spectrum of X-irradiated aqueous solution containing dThd and 4-PyOBN in the presence of O_2 is depicted in Figure 2A. Figure 2B shows the ESR spectrum that was observed in the fraction separated by Sep-Pak C_{18} cartridge with a 10% methanol solution. Figure 2C is the ESR spectrum from spin adduct recovered in the fraction eluted by a 50% methanol solution. Both spectrum B and spectrum C of Figure 2 exhibited a secondary doublet unaccompanied by hfs due to a nitrogen atom. From this result, it is concluded that no N-centered radicals were trapped by 4-PyOBN. Figure 2B seems to correspond to Figure 1B. Figure 2C seems to correspond to Figure 1C. To get more convincing evidence, we exclusively produced a radical at the C5 position of the base moiety and forcibly added it to MNP and 4-PyOBN in the next experiment, and the resulting spectrum was compared with those in Figures 1 and 2.

Spin Trapping of Hydrated Electron Induced TMP Radicals by MNP and 4-PyOBN. Hydrated electrons, e_{aq}^-, were prepared by X irradiation of aqueous solution containing the OH radical scavenger sodium formate in the absence of O_2. The hydrated electrons react with dThd followed by protonation to form a 5,6-dihydrothymidin-5-yl radical (Bernhard, 1983).



(7)

This selective formation of the C5-centered radical was useful in obtaining conclusions about what kind of ESR spectrum was given by the adduct of the 5-yl radical and MNP or 4-PyOBN. No disturbance by undesirable side products from the spin-trapping reagent itself was observed. Figure 3A shows the ESR spectrum of the adduct of the 5-yl radical and MNP. Figure 3B shows that from a combination of dThd and 4-PyOBN. Figure 3A was almost identical with Figure 1C. This indicates that the assignment of the spectrum shown in Figure 1C to the trapping of the C5-centered radical in the base moiety was correct. In addition, Figure 3B is quite similar to Figure 2C. This result proves that spin trapping of the

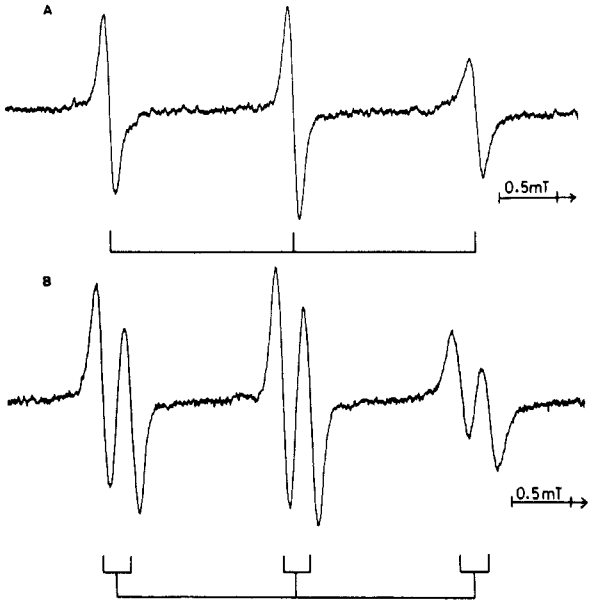


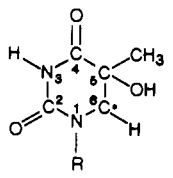
FIGURE 3: (A) ESR spectrum obtained after an Ar-saturated aqueous solution containing dThd (20 mM), MNP (10 mM), and sodium formate (1 M) was exposed to 4 kGy of X-rays at 77 K and thawed at 45 °C and (B) ESR spectrum obtained after an Ar-saturated aqueous solution containing dThd (20 mM), 4-PyOBN (2 mM), and sodium formate (1 M) was exposed to 4 kGy of X-rays at 77 K and thawed at 45 °C.

Table I: Primary and Secondary Hyperfine Couplings of Spin-Trapped dThd and DNA Radicals Formed by Reactions with MNP and 4-PyOBN[a]

	MNP			4-PyOBN		
compd	a_N (mT)	a_H^β (mT)	a_N^β (mT)	a_N (mT)	a_H^β (mT)	radical structure
dThd	1.58			1.495	0.23	I
	1.44		0.28	1.51	0.10	II
	1.67	1.30 (2H)		1.525	0.31	III
DNA	1.59					I
	1.46		0.29			II
	1.70	1.34 (2H)				III

[a] a_N denotes a primary ^{14}N splitting. a_H^β and a_N^β denote secondary splitting by a β-proton and β-nitrogen, respectively.

C5-centered radical by 4-PyOBN resulted in the generation of an ESR spectrum consisting of a primary triplet of 1.495 mT and a secondary doublet of 0.23 mT (see Table I). The spectrum isolated by a Sep-Pak C_{18} cartridge with a 10% methanol solution (Figure 2B) is, therefore, considered to correspond to the spectrum shown in Figure 1B. Since no evidence for the presence of an N-centered radical in dThd could be given, spin trapping at the C6 position, where the free radical is induced by OH addition to the C5 position of the double bond, is the most probable explanation (structure II) for Figures 1B and 2B.



II

The H-abstraction radical at the C1′ position of the sugar moiety can also be considered to be another candidate. If, for example, the C1′-centered radical is trapped by MNP and 4-PyOBN, the N1 atom of the base moiety is present at the β-position for MNP and the γ-position for 4-PyOBN, respectively, and is consistent with the observed spectra. However, trapping of the C1′-centered radical is less likely. In the case of dThd, 80–90% of the OH radicals react with the base moiety. Thus, only 10–20% of the OH radicals can react with the deoxyribose portion of the molecule (Ward & Kuo, 1973). Only about 10% of the OH radicals, which react with the sugar moiety, can abstract a hydrogen atom at the C1′ position (Isilder et al., 1981). As a result, 1–2% of the OH radicals react to produce a free radical at the C1′ position, whereas half of the remaining 80–90% of the OH radicals are added to the C5 position of the base moiety to produce a C6-centered radical (Têoule & Cadet, 1975). Therefore, it is more probable that the spectrum in Figure 1B, obtained by MNP, and the spectrum in Figure 2B, obtained by 4-PyOBN, are attributable to the trapping of the 6-yl radical of the thymine base.

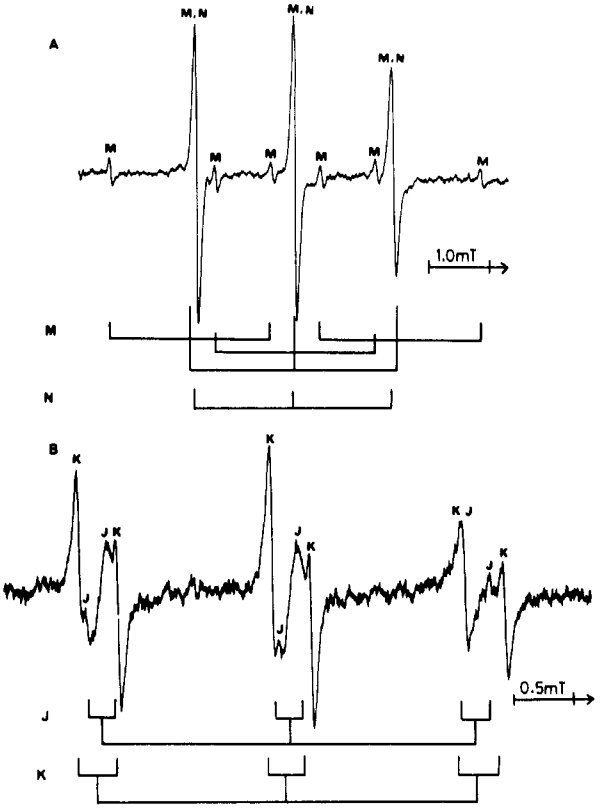
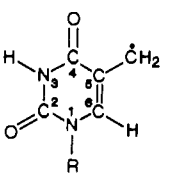


FIGURE 4: (A) ESR spectrum obtained after γ-irradiated solid dThd was dissolved in an Ar-saturated aqueous solution containing MNP (20 mM) and sodium formate (1 M) and (B) ESR spectrum obtained after γ-irradiated solid dThd was dissolved in an Ar-saturated aqueous solution containing 4-PyOBN (220 mM) and sodium formate (1 M).

Spin Trapping of Free Radicals Produced in γ-Irradiated Solid dThd by MNP and 4-PyOBN. As previously described, one more radical was trapped by MNP when spin trapping of dThd was done in the absence of oxygen (Kuwabara et al., 1981a; Zhang et al., 1983). This radical was a 5-methyleneuracil radical formed by H abstraction at the 5-methyl group of the base moiety. An ESR study of irradiated solid dThd has shown that irradiation of dThd in the solid state preferentially produces a 5-methyleneuracil radical (structure III) together with the above-mentioned 5,6-dihydro-



III

thymidin-5-yl radical (Bernhard, 1983). Since we already have

spectra characterized as trappings of the 5-yl radical by 4-PyOBN and by MNP (Figures 2C and 3B), the application of the trapping by 4-PyOBN, as well as that by MNP, to solid-state radicals will give information about what kind of spectrum is obtained from the trapping of the 5-methyleneuracil radical (structure III). The result is shown in Figure 4. The ESR spectrum shown in Figure 4A was obtained from the spin trapping of free radicals produced in solid dThd by MNP with a specially designed U-tube. Signal M shows a very large splitting of 5.94 mT from the peak of the lowest field to the peak of the highest field. Furthermore, it was observed that this signal consists of a primary triplet of 1.67 mT and a secondary 1:2:1 triplet of 1.30 mT, although the inner components are somewhat obscured by ESR absorption from the other adducts. These characteristics result from the interaction of the spin with two protons at the β-position. Therefore, this can be explained by the spin trapping of the $^{\bullet}CH_2-$ radical by MNP (Lagercranz & Setaka, 1974; Rustgi & Riesz, 1978). This is also the only ESR spectrum that can be identified without ambiguity. The 5-methyleneuracil radical (structure III) formed by H loss from the 5-methyl group of the thymine base is consistent with this signal. A quite similar spectrum has already been reported for dThd (Kuwabara et al., 1981a).

Another signal marked N is observed in Figure 4A. This signal is associated with the 5-yl radical and corresponds almost exactly to Figures 1C and 3A. This again leads to the conclusion that the trapping of the 5-yl radical by MNP can be associated with a spectrum consisting of only a primary triplet.

The result obtained by using 4-PyOBN is shown in Figure 4B. This spectrum consists of two sets of signals marked J and K. Set J has a primary triplet of 1.50 mT, which further splits into a doublet of 0.22 mT. These hfs constants are almost equal to those obtained from Figures 2C and 3B. This is regarded as the trapping of the 5-yl radical by 4-PyOBN. Set K, consisting of a primary triplet of 1.525 mT, which further splits into a doublet of 0.31 mT, can, therefore, be associated with the trapping of the $^{\bullet}CH_2-$ radical. All hfs constants are listed in Table I.

ESR Spectra of the Spin Adducts of DNA and MNP. ESR spectra recorded after irradiation of an N_2O-saturated aqueous solution containing DNA and MNP are depicted in Figure 5. Figure 5A shows a spectrum immediately after irradiation. This was broad and insufficient for analyzing the site of the damage. Figure 5B shows an ESR spectrum recorded at 40 °C after the DNA was digested by DNase I. It can clearly be seen that the spectrum is well resolved and analyzable. This is due to the fact that the size of the DNA was reduced to oligonucleotides by digestion, resulting in free rotation of the spin adducts. This spectrum seems to consist of several sets of signals from different spin adducts, but we could not separate them by chromatography because DNase I digested the DNA to produce different sizes of oligonucleotides.

Several investigators have reported results obtained from spin trapping of OH-induced radicals in nucleic acid constituents, especially for nucleosides and nucleotides (Joshi et al., 1976; Kominami et al., 1976, 1977; Kuwabara et al., 1982, 1986; Inanami et al., 1986). The ESR spectrum characteristics of each molecule are given in their papers. Comparison of the ESR spectrum of DNA with each of these spectra suggests that the spectra obtained from dThd and TMP are most similar to that obtained from DNA. This is further supported by the fact that the ESR spectrum of yeast RNA was different from that of DNA (Figure 5C).

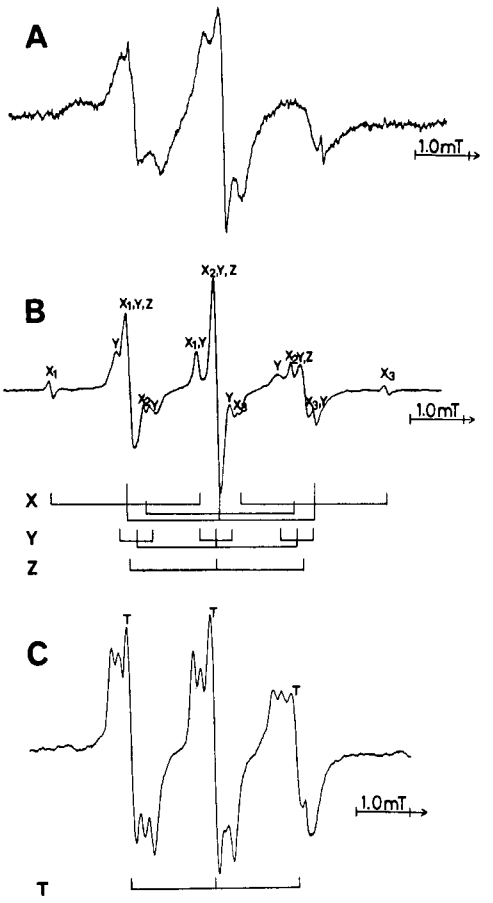


FIGURE 5: (A) ESR spectrum obtained immediately after an N_2O-saturated aqueous solution containing DNA (14 mg/mL) and MNP (0.8 mg/mL) was exposed to 2.7 kGy of X-rays, (B) ESR spectrum recorded at 40 °C after 100 μg of DNase I was added to the solution and incubated at 37 °C for 30 min, and (C) ESR spectrum recorded at 40 °C after an N_2O-saturated aqueous solution containing yeast RNA (14 mg/mL) and MNP (0.8 mg/mL) was exposed to 2.7 kGy of X-rays and treated by incubation with RNase A (100 μg/mL) at 37 °C for 30 min.

Signal X in Figure 5B shows a very large splitting of 6.08 mT from the peak of the lowest field to the peak of the highest field. Furthermore, this signal consists of a primary triplet of 1.7 mT and a secondary 1:2:1 triplet of 1.34 mT. Tis corresponds almost exactly to the spectrum labeled M in Figure 4A. A radical consistent with this signal is the 5-methyleneuracil radical (structure III), the precursor for 5-(hydroxymethyl)uracil.

The signal labeled Y is an ESR spectrum consisting of a primary triplet of 1.46 mT which further splits into a secondary 1:1:1 triplet of 0.29 mT. This is also attributable to the spin trapping of a radical at the thymine moiety, because a quite similar spectrum was obtained in dThd (Figure 1B). The site for the radical formation is the C6 position, where the free radical is induced by OH addition to the C5 position of the double bond of the thymine base (5-hydroxy-5,6-dihydrothymin-6-yl radical; structure II).

In Figure 5B there is a third signal labeled Z. This signal is an ESR spectrum consisting of only a primary triplet. Similar spectra have been given for dThd and TMP, not only in the present report (Figures 1C, 3A, and 4A) but also in previous reports (Joshi et al., 1976, Kominami et al., 1977; Kuwabara et al., 1982). However, the ESR spectrum of yeast RNA also contained a signal similar to that marked Z (signal T in Figure 5C). Hyperfine coupling of ^{14}N was 1.59 mT for DNA and was almost the same as that from dThd and TMP, whereas the hyperfine coupling was 1.53 mT for RNA and

was quite similar to that from purine nucleosides and their homopolymers (Kuwabara et al., 1986). This means that signal Z can be regarded as arising from a spin-trapped radical at the thymine base moiety of DNA and can thus be assigned to the 6-hydroxy-5,6-dihydrothymin-5-yl radical (structure I). As for the spectrum consisting of a primary triplet with 1.53 mT for RNA, this was assigned to the H-abstraction radical at the C4′ position of the sugar moiety, which is regarded as a precursor for strand breaks (Kuwabara et al., 1986; Inanami et al., 1986). The hfs constants obtained for DNA are listed in Table I.

Discussion

This study was carried out to obtain evidence for the presence of precursor radicals that lead to biological damage of DNA induced by reactions with the OH radical, one of the activated oxygens. We first studied the spin trapping of dThd, a constituent of DNA, by the use of both MNP and 4-PyOBN spin-trapping reagents in combination with liquid chromatography. The selective formation of radicals in dThd, followed by chromatographic analysis, made it possible for us to infer that at least three radical species were produced in dThd. The ESR spectrum shown in Figure 1B had a secondary 1:1:1 triplet hyperfine structure. A spin-trapping experiment using 4-PyOBN clearly indicated that no N-centered radicals were formed by the reaction of dThd with OH radicals. The most probable site for the radical formation is the C6 position, where the free radical is induced by OH addition to the C5 position of the double bond (structure II).

The ESR spectrum shown in Figure 1C and ESR spectrum M in Figure 4A were attributed to the C5-centered radical produced by OH addition to the 5,6 double bond (structure I) and the 5-methyleneuracil radical produced by H abstraction at the methyl group of the base (structure III), respectively, without any ambiguity.

For DNA, the spin-trapping method was employed in an improved manner, utilizing pretreatment of the DNA solution by sonolysis and enzymatic digestion of the adduct of DNA and the spin-trapping reagent. Thus, an ESR spectrum with well-resolved and analyzable hfs could be obtained. Since the enzyme digested the DNA to different sizes of oligonucleotides, the spin adducts could not separated by chromatography on the basis of the adduct form. Nevertheless, comparison of the ESR spectrum with the spectra obtained from RNA, as well as from DNA constituents, suggested that free radicals trapped by MNP were exclusively induced at the thymine moiety.

The ESR spectrum obtained from DNA is shown in Figure 5B. At least three sets of signals are contained in this figure. Set X was attributed to the trapping of an H-abstraction radical at the methyl group of the thymine base (structure III) and was regarded as the precursor of 5-(hydroxymethyl)uracil. Set Y was assigned to the 6-yl radical of the thymine base (structure II) and was regarded as the precursor for 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol), which is well-known as a cause of thymine damage. Set Z was attributed to the trapping of the C5-centered radical produced by OH addition to the 5,6 double bond of the thymine base (structure I) and was also regarded as the precursor of the 6-hydroxy-5,6-dihydrothymine.

When another spin trap, 4-PyOBN, was employed, the trapping of a radical having structure I gave the ESR spectra shown in Figures 2C and 3B. The trapping of a radical having structure II gave the ESR spectrum shown in Figure 2B. The trapping of a radical having structure III gave the ESR spectrum labeled K in Figure 4B. The use of two kinds of spin-trapping reagents, as well as isolation of the spin adducts by chromatography and the selective formation of radicals under various irradiation conditions, enabled us to correlate each spectrum to the corresponding radical.

This study using a spin-trapping method only elucidated the presence of precursors leading to thymine base damage, although OH radicals react with DNA not only at the thymine base but also at three other bases and the sugar–phosphate backbone. This result might be due to the higher stability of spin adducts at the thymine base than at the other sites of DNA. The high reactivity of OH radicals to the thymine base might also be another factor responsible for the localization of the spin adducts. In fact, studies of the reactions of OH radicals with DNA have shown that the thymine base is particularly susceptible to OH radical attack (Scholes et al., 1960; Têoule & Cadet, 1978). Actually, some oxidative conversions, thymine glycol, 6-hydroxy-5,6-dihydrothymine, and 5-(hydroxymethyl)uracil, were found to be major sources of DNA damage in irradiated cultured mammalian cells (Frenkel et al., 1981a,b, 1985; Teebor et al., 1982, 1984; Leadon & Hanawalt, 1983; Breimer & Lindahl, 1985), and the relationship between the excision repair of thymine base damage and the recovery of cells from serious injury is now a matter of intrest to many investigators (Lindahl, 1982).

The employment of two spin traps for the spin-trapping study enabled us to discover the relationship between hfs constants in the ESR spectra obtained by nitrone spin traps and the structure of the radicals, although either trap alone was unsuitable for the identification of the structure of the radicals. MNP cannot be utilized for a spin-trapping study at the cellular level because of its serious toxic effect, whereas nitrone spin traps have relatively low toxicity (Morgan et al., 1985). Therefore, if both kinds of spin traps are first used on pure molecules such as DNA, RNA, and proteins and then nitrone compounds alone are adapted to experiments at the cellular level as a spin trap, it will become possible for us to trap the free radical intermediates and to identify the sites of radical formation in the irradiated cells.

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A Study of Side Reactions Occurring during Synthesis of Oligodeoxynucleotides Containing O^6-Alkyldeoxyguanosine Residues at Preselected Sites†

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ABSTRACT: As part of our studies on the molecular mechanisms of mutation by carcinogens we have synthesized 12 oligonucleotides (15-mers) containing an O^6-alkylguanine residue at a preselected position for use as primers in the enzymatic synthesis of biologically active DNA. Ten of these oligonucleotides are derived from a minus strand sequence carrying the modified nucleotide in the third codon of gene G of bacteriophage ΦX174 DNA. Two others are derived from plus strand sequences carrying the modification in the 12th codon of the human Ha-*ras* protooncogene. During this work several potentially serious side reactions, which could complicate interpretation of mutagenesis data, were observed. This paper describes a detailed study of these reactions. Since we were unable to avoid undesirable side products, we developed simple chromatographic methods for detecting and removing them.

O^6-Alkylguanine moieties in DNA have received considerable attention ever since Loveless suggested that they were important premutagenic and precarcinogenic lesions (Loveless, 1969). As part of our studies on the molecular mechanisms of mutation by carcinogens we have synthesized 12 oligonucleotides (15-mers) containing an O^6-alkyl group at a preselected position for use as primers in the enzymatic synthesis of biologically active DNA. Ten of these oligonucleotides are derived from a minus strand sequence carrying the modified nucleotide in the third condon of gene G of bacteriophage ΦX174 DNA. Two others are derived from plus strand sequences carrying the modification in the 12th condon of the

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