performing the amino acid and sequence analysis.

References

Ahrland, S. (1979) *Pure Appl. Chem. 51*, 2019.
Archer, D. W., & Monk, C. B. (1964) *J. Chem. Soc.*, 3117.
Auld, D. S. (1987) in *Enzyme Mechanisms* (Page, M. I., & Williams, A., Eds.) 256 ff, Royal Society of Chemistry, Herts, U.K.
Auld, D. S., & Vallee, B. L. (1970) *Biochemistry 9*, 4352.
Auld, D. S., & Vallee, B. L. (1971) *Biochemistry 10*, 2892.
Auld, D. S., Latt, S. A., & Vallee, B. L. (1972) *Biochemistry 11*, 4994.
Auld, D. S., Larsen, K. S., & Vallee, B. L. (1986) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., & Zeppezauer, M., Eds.) pp 133–154 Birkhäuser, Boston.
Bazzone, T. J., Sokolovsky, M., Cueni, L. B., & Vallee, B. L. (1979) *Biochemistry 18*, 4362.
Bologni, L., Sabatini, A., & Yacca, A. (1983) *Inorg. Chim. Acta 69*, 71.
Coleman, J. E., & Vallee, B. L. (1960) *J. Biol. Chem. 235*, 390.
Cox, D. J., Bovard, F. C., Bargetzi, J.-P., Walsh, K. A., & Neurath, H. (1964) *Biochemistry 3*, 44.
Dixon, M., & Webb, E. C. (1964) *Enzymes*, pp 118–145, Academic Press, New York.
Fulton, J. W., & Swinehart, D. F. (1954) *J. Am. Chem. Soc. 76*, 864.
Geoghegan, K. F., Holmquist, B., Spilburg, C. A., & Vallee, B. L. (1983) *Biochemistry 22*, 1847.
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., & Singh, R. M. M. (1966) *Biochemistry, 5*, 467.
Gubeli, A. O., & Ste-Marie, J. (1967) *Can. J. Chem. 45*, 827.
Hanahan, D., & Auld, D. S. (1980) *Anal. Biochem. 108*, 86.
Hirose, J., Noji, M., Kidani, Y., & Wilkins, R. G. (1985) *Biochemistry 24*, 3495.
Hirose, J., Ando, S., & Kidani, Y. (1987) *Biochemistry 26*, 6561.
Holmquist, B. (1988) *Methods Enzymol. 158*, 6.
Larsen, K. S., Auld, D. S., & Vallee, B. L. (1986) *Biol. Chem. Hoppe-Seyler 367*, 367.
Lipscomb, W. N. (1974) *Tetrahedron 30*, 1725.
Lobb, R. R., & Auld, D. S. (1984) *Experientia 40*, 1197.
Perkins, D. J. (1953) *Biochem. J. 55*, 649.
Perrin, D. D. (1962) *J. Chem. Soc.*, 4500.
Pêtra, P. H., & Neurath, H. (1969) *Biochemistry 8*, 5029.
Pollack, S. E., & Auld, D. S. (1982) *Anal. Biochem. 127*, 81.
Rees, D. C., Lewis, M., & Lipscomb, W. N. (1983) *J. Mol. Biol. 168*, 367.
Schorsch, G. (1964) *Bull. Soc. Chim. Fr.*, 1449.
Schorsch, G. (1965) *Bull. Soc. Chim. Fr.*, 988.
Sekine, T. (1965) *Acta Chem. Scand. 19*, 1526.
Sillén, S. G., & Martell, A. E. (1964) *Spec. Publ.—Chem. Soc. No. 17*, 61–63.
Stephens, R. S., Jentoft, J. E., & Bryant, R. G. (1974) *J. Am. Chem. Soc. 96*, 8041.
Shoham, G., Christianson, D. W., & Oren, D. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 684.
Vallee, B. L., & Neurath, H. (1954) *J. Am. Chem. Soc. 76*, 5006.
Vallee, B. L., Rupley, J. A., Coombs, T. L., & Neurath, H. (1960) *J. Biol. Chem. 235*, 64.
Williams, A. C., & Auld, D. S. (1986) *Biochemistry 25*, 94.

Evidence for the Formation of Strand-Break Precursors in Hydroxy-Attacked Thymidine 5′-Monophosphate by the Spin Trapping Method

Mikinori Kuwabara,* Wakako Hiraoka, and Fumiaki Sato

Department of Radiation Biology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

Received March 8, 1989; Revised Manuscript Received July 11, 1989

ABSTRACT: A method combining spin trapping, ESR, and HPLC was employed to obtain evidence for the formation of sugar radicals in OH-attacked TMP with special emphasis on the detection of strand-break precursors of DNA. OH radicals were produced by irradiating an N_2O-saturated aqueous solution with X-rays. When an N_2O-saturated aqueous solution containing TMP and a spin trapping reagent, MNP, was irradiated with X-rays, it was estimated on the basis of theoretical calculations using rate constants that 94% of the TMP radicals were induced by OH radicals. Since several spin adducts between TMP radicals and MNP, as well as the byproducts of the spin trapping reagent itself, were produced, reverse-phase HPLC was used to separate them. The presence of six spin adducts was confirmed by ESR examination. Further examination of these spin adducts by UV absorbance spectrophotometry showed the presence of a chromophore at 260 nm in three adducts. Since a gradual increase in the release of unaltered base from these adducts was observed when they were allowed to stand for 0–22 h at room temperature, they could be regarded as the spin adducts of sugar radicals and MNP. ESR spectra from the spin adducts were consistent with hydrogen abstraction radicals at the C1′, C4′, and C5′ positions of the sugar moiety. These radicals appeared to be precursors of AP sites and strand breaks. In addition to these spin adducts, ESR spectra that were consistent with the spin adducts of base radicals (the C5 and C6 radicals) and MNP were observed.

Hydroxy radicals, which are the most reactive species among the active oxygens, react with DNA to induce strand breaks, AP[1] sites, and base modification (Têoule, 1987; von Sonntag, 1987; Teebor et al., 1988). TMP is a DNA con-

0006-2960/89/0428-9625$01.50/0

stituent, and its oxidative base products induced by OH radicals have been identified by chemical and biochemical methods. The specific repair enzymes which recognize these modified thymine bases have also been identified (Lindahl, 1982; Teebor & Frenkel, 1983; Teebor et al., 1988). In a previous paper we identified thymine base radicals, which appear to be precursors of oxidative thymine base damage, in OH-attacked DNA (Kuwabara et al., 1987). Until now, the majority of the papers dealing with precursor radicals of oxidative thymine damage have only used dThd and TMP as models of DNA, though for our previous papers we used DNA itself (Joshi et al., 1976; Kominami et al., 1977; Kuwabara et al., 1982). While there are several papers concerning OH-induced thymine base radicals, only one paper, in which an alkoxy radical at the sugar moiety was reported by ESR investigation of the γ-irradiated single crystal of dThd at 4.2 K, is available (Box & Budzinski, 1975). We have recently reported the observation of OH-induced sugar radicals in 5′-dGMP and 5′-dAMP, which are other DNA constituents (Hiraoka et al., 1989), but it is also important to identify the sugar radicals in OH-attacked TMP, because this is the most typical constituent of DNA. In the present paper this purpose was achieved by employing a spin trapping method using MNP as a spin trap. OH radicals were generated by irradiating an N_2O-saturated aqueous solution with X-rays. Since several spin adducts between TMP radicals and MNP, as well as the byproducts due to MNP itself, were produced, these were separated by reverse-phase HPLC. Of six spin adducts separated, three had a chromophore at 260 nm. Reexamination by HPLC proved that each of these adducts had an unaltered thymine base, indicating that the spin trapping undoubtedly occurred at the sugar moiety. From analysis of the ESR spectra, precursor radicals of AP sites and precursor radicals of strand breaks were identified. The assignment of spin adducts was further confirmed by similar experiments with ddTMP. A preliminary paper on this work has already been published (Kuwabara, 1988).

Experimental Procedures

Materials

TMP and 2′,3′-dideoxythymidine were purchased from Sigma Chemical Co. MNP and dicyclohexylcarbodiimide were from Aldrich Chemical Co. Barium 2-cyanoethyl phosphate dihydrate was from Dojindo Laboratories (Kumamoto, Japan). TEAA, which was used as an elution solution for HPLC, was prepared by mixing equimolar amounts of acetic acid and triethylamine and adjusted to pH 6.5. ddTMP was prepared according to the method of Tener (1961). 2′,3′-Dideoxythymidine was first converted to 2′,3′-dideoxythymidine 5′-(2-cyanoethylphosphate) by reaction between 2′,3′-dideoxythymidine and 2-cyanoethyl phosphate that was catalyzed by dicyclohexylcarbodiimide in dry pyridine. Ethyl cyanide was then removed from this ester by ammonium hydroxide at 60 °C for 75 min. Finally, ddTMP was purified by DEAE-cellulose column chromatography.

Methods

Spin Trapping of TMP Radicals. Spin trapping was carried out essentially according to the method of Janzen (1971). To generate OH radicals, an N_2O-saturated aqueous solution was irradiated with a dose of 5.4 kGy of X-rays. This solution contained N_2O, TMP, and MNP at concentrations of 27, 5, and 10 mM, respectively. Irradiation of the solution with X-rays was carried out by operating a Toshiba X-ray machine at 180 kV and 25 mA, generating 1.52 mM OH radicals, 1.49 mM e_{aq}^-, and 0.31 mM H radicals. OH radicals react competitively with TMP and MNP, accompanied by the generation of TMP radicals and the degradation of MNP. hydrated electrons react competitively with TMP, MNP, and N_2O. The reaction of hydrated electrons (e_{aq}^-) with N_2O converts them to OH radicals, which further react competitively with TMP and MNP. Hydrogen radicals also react competitively with TMP and MNP. By use of the reaction rate constants of these molecules with OH, e_{aq}^-, and hydrogen radicals, the concentration of the OH-induced TMP radicals was calculated to be 0.78 mM. Similarly, the concentrations of e_{aq}^--induced TMP radicals and hydrogen radical induced TMP radicals were calculated to be 0.02 and 0.03 mM, respectively. As a result, 94% of the TMP radicals were calculated to be OH-induced. Furthermore, 25% of the water radicals were calculated to react with TMP, and 75% of them were calculated to react with MNP (see details under Results and in Table I). Thus, it was concluded that the majority of TMP radicals were OH-induced. The TMP radicals (R•) react further with MNP to produce spin adducts:

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

In the case of ddTMP, the experimental conditions employed were the same as those described above.

Separation of Spin Adducts by Reverse-Phase HPLC in the Ion-Suppression Mode. Since irradiation with X-rays produced several spin adducts between TMP (or ddTMP) radicals and MNP, as well as byproducts derived from MNP itself, these were separated by reverse-phase HPLC in the ion-suppression mode. TEAA was used as a paired-ion reagent for the phosphate group of TMP (or ddTMP) as described by Ip et al. (1985). After 1 mL of the solution was irradiated with X-rays, it was injected into a μBondapak C_{18} column (0.39 × 30 cm, Waters Associates) attached to a Tosoh HPLC system (CCP and 8000). Elution was carried out with 0.02 M TEAA (pH 6.5) by a linear-gradient program at a rate of 0.33% methanol/min for 15 min and followed by a linear-gradient program at a rate of 1.5% methanol/min for 30 min. The rate of elution was 1.0 mL/min. The eluted solution was monitored for UV absorbance at 260 nm with 1.28 aufs. Each peak was collected, and the presence of the spin adduct was ascertained by ESR spectroscopy. In the case of UV spectrophotometry, since TEAA had a strong chromophore at wavelengths under 240 nm, which obscured the UV absorbance of the spin adduct, the solution was freeze-dried to remove volatile TEAA and again dissolved in 2.5 mL of triply distilled water.

Measurements of ESR and UV Absorbance Spectra. The separated spin adducts were characterized by ESR and UV spectrometry. ESR measurements were made on a JEOL ME X-band spectrometer. 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 10 mW. The α, β, and γ positions of magnetic nuclei in the spin adduct were determined with respect to the unpaired electron on the nitrogen of the nitroxide according to the definition of Riesz and Rustgi (1982). UV absorbance spectra were recorded by a Hitachi 340 spectrophotometer.

[1] Abbreviations: AP, apurinic/apyrimidinic; aufs, absorbance units full scale; ddTMP, 2′,3′-dideoxythymidine 5′-monophosphate; e_{aq}^-, hydrated electron; dThd, thymidine; ESR, electron spin resonance; HPLC, high-performance liquid chromatography; MNP, 2-methyl-2-nitrosopropane; *t*-Bu, *tert*-butyl; TEAA, tetraethylammonium acetate; TMP, thymidine 5′-monophosphate; 3′-UMP, uridine 3′-monophosphate; UV, ultraviolet.

Table I: Chemical Reaction Modes of OH Radicals, Hydrated Electrons, and Hydrogen Radicals with TMP and MNP[a]

$OH^{\bullet}$ (1.52 mM)	MNP $\xrightarrow{k_1}$	byproducts (0.99 mM; 67.5%)	(1)
	TMP $\xrightarrow{k_2}$	TMP radicals (0.53 mM; 32.5%)	(2)
e_{aq}^- (1.49 mM)	MNP $\xrightarrow{k_3}$	byproducts (0.71 mM; 47.7%)	(3)
	TMP $\xrightarrow{k_4}$	TMP radicals (0.02 mM; 1.3%)	(4)
	N_2O $\xrightarrow{k_5}$	OH radicals (0.76 mM; 51.0%)	(5)
	MNP $\xrightarrow{k_1}$	byproducts (0.51 mM)	(6)
	TMP $\xrightarrow{k_2}$	TMP radicals (0.25 mM)	(7)
$H^{\bullet}$ (0.31 mM)	MNP $\xrightarrow{k_6}$	byproducts (0.28 mM; 90.3%)	(8)
	TMP $\xrightarrow{k_7}$	TMP radicals (0.03 mM; 9.7%)	(9)

[a] $k_1 = 5.0 \times 10^9$ M^{-1} s^{-1} (Greenstock, 1978), $k_2 = 5.3 \times 10^9$ M^{-1} s^{-1} (Dorfman & Adams, 1973), $k_3 = 2.2 \times 10^{10}$ M^{-1} s^{-1} (Kuwabara et al., unpublished results), $k_4 = 1.5 \times 10^9$ M^{-1} s^{-1} (Anbar et al., 1973), $k_5 = 8.7 \times 10^9$ M^{-1} s^{-1} (Anbar et al., 1973), $k_6 = 1.9 \times 10^9$ M^{-1} s^{-1} (Greenstock, 1978), and $k_7 = 2.3 \times 10^8$ M^{-1} s^{-1} (Anbar et al., 1975).

Kinetic Measurements of the Release of Unaltered Thymine Base from the Spin Adducts. One hundred microliters of an N_2O-saturated aqueous solution containing TMP and MNP was irradiated to a dose of 5.4 kGy of X-rays and applied to the HPLC system. The chromatographic system and elution conditions were the same as those described above. After each spin adduct having an ESR signal was once more purified by HPLC under the same conditions, the spin adduct was allowed to stand for 0–22 h at room temperature and then injected into the HPLC column to find the unaltered thymine base. The amounts of the released base and concomitant decreases of the spin adduct were measured as a function of time.

Results

Estimation of the Concentration of OH-Induced TMP Radicals. Saturation of the aqueous solution with N_2O gas gave rise to a 27 mM N_2O solution. Irradiation of the aqueous solution with 5.4 kGy of X-rays resulted in the formation of 1.52 mM OH radicals, 1.49 mM e_{aq}^-, and 0.31 mM hydrogen radicals. These values were calculated from the yields of OH radicals (2.7), e_{aq}^- (2.65), and hydrogen radicals (0.35) per 100 eV of energy absorption of X-rays (Henglein et al., 1969). In aqueous solution in which 10 mM MNP, 5 mM TMP, and 27 mM N_2O were dissolved, the fractions of OH radicals that reacted with the solutes were calculated, by use of each rate constant, to be 67.5% for MNP and 32.5% for TMP according to reactions 1 and 2 in Table I. Hydrated electrons (e_{aq}^-) reacted with N_2O as well as MNP and TMP (47.7%, 1.3%, and 51.0%, respectively; reactions 3, 4, and 5). The reaction of e_{aq}^- with N_2O converted them to OH radicals, which further reacted competitively with MNP and TMP (reactions 6 and 7). As for hydrogen radicals, they reacted competitively with MNP (90.3%) and TMP (9.7%) as shown in reactions 8 and 9.

By use of the reaction modes shown in Table I, the total concentration of TMP radicals was estimated to be 0.83 mM (0.53 + 0.25 + 0.02 + 0.03 mM). The concentration of the byproducts was estimated to be 2.49 mM. These results indicated that one-fourth of the water radicals reacted with TMP to give rise to TMP radicals and that three-fourths of the water radicals reacted with MNP to give rise to byproducts. From these results it was concluded that the majority of TMP radicals were OH-induced (94% = 0.78/0.83 × 100), but since

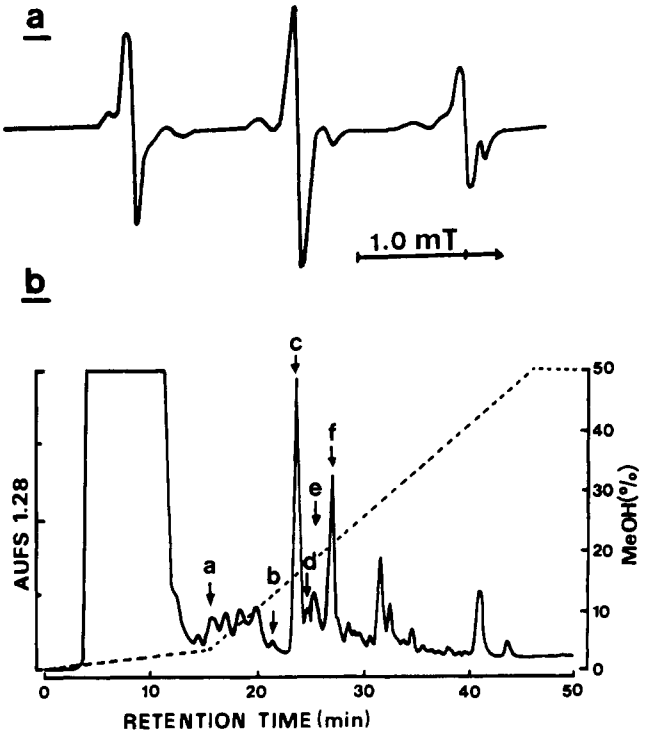

FIGURE 1: (a) ESR spectrum of spin-trapped radicals from an N_2O-saturated aqueous solution containing 5 mM TMP and 10 mM MNP immediately after irradiation with 5.4 kGy of X-rays. (b) Elution profile after 1 mL of N_2O-saturated aqueous solution containing TMP and MNP was irradiated with X-rays and separated by reverse-phase HPLC in the ion-suppression mode. Separation conditions are described in the text.

irradiation with X-rays produced not only several spin adducts but also byproducts, a technique to isolate the spin adducts from the byproducts and to separate the spin adducts into their components had to be introduced.

Separation of Spin Adducts by the HPLC–ESR System and Examination by UV Absorbance Spectrophotometry. Figure 1a shows the ESR spectrum obtained immediately after irradiation of an N_2O-saturated aqueous solution containing TMP and MNP. Since overlapping of the signals due to several spin adducts was observed, HPLC with a reverse-phase column was employed to separate and identify the spin adducts in the ion-suppression mode, 0.02 M TEAA being used as the paired-ion reagent for the phosphate group of TMP. A chromatogram was obtained by UV absorbance at 260 nm (Figure 1b). After undamaged TMP molecules were eluted as a major constituent of the solutes, many peaks appeared in the chromtographic profile. When each peak was examined by the ESR technique, a total of six signals was detected from the peaks denoted a–f. No signals from the other peaks were found. The UV absorbance spectra and ESR spectra obtained from each peak are shown in Figure 2. Of the six spin adducts, the spin adducts belonging to peaks a, b, and d showed maximum absorbance at 260 nm, and the spin adducts denoted c, e, and f showed the absence of a chromophore at 260 nm. Therefore, as a temporary measure, the spin adducts of a, b, and d were regarded as those of the sugar radicals, and the spin adducts of c, e, and f were regarded as those of the base radicals.

Kinetics of Unaltered Thymine Release from the Spin Adducts. As a result of studies by von Sonntag and Schulte-Frohlinde (1978), the sugar radicals have been inferred to be linked to the release of an unaltered base after several radical reactions. Although the sugar radicals are initially trapped by MNP, the release of the unaltered base from the

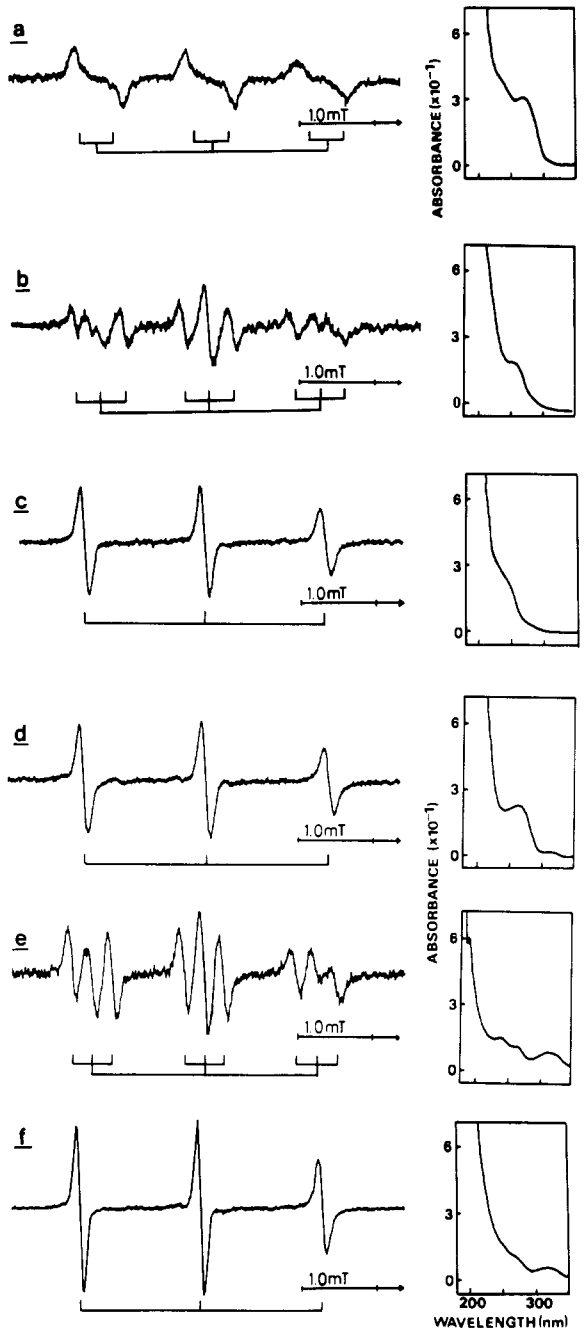

FIGURE 2: ESR spectra (left) and UV absorbance spectra (right) obtained from peaks a–f in Figure 1.

separated spin adduct may be observed if the separated spin adduct is allowed to stand for an appropriate interval at room temperature. This technique was utilized to judge whether each spin adduct had an unaltered thymine base. If the release of an unaltered base from the separated spin adduct was observed, this adduct would be taken to be that of the sugar radical, whereas no release of an unaltered base from the separated spin adduct would give proof of spin trapping at the base moiety. The elution profile shown in Figure 3a was obtained after 100 μL of an N_2O-saturated aqueous solution containing only TMP was irrdiated with a dose of 5.4 kGy of X-rays and applied to the HPLC column. The release of an unaltered thymine base was observed. Figure 3b shows the HPLC elution profile after 100 μL of an N_2O-saturated aqueous solution containing TMP and MNP was irradiated with X-rays. In addition to the unaltered thymine base, the elution of the spin adducts between TMP radicals and MNP

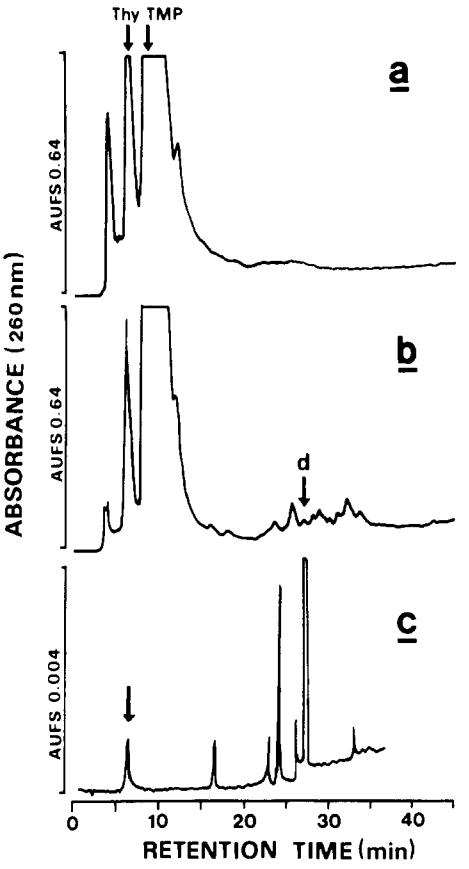

FIGURE 3: (a) Elution profile after 100 μL of an N_2O-saturated aqueous solution containing 5 mM TMP alone was irradiated with X-rays and separated by reverse-phase HPLC in the ion-suppression mode. The elutions of unaltered thymine and TMP are indicated by arrows. (b) Elution profile after an N_2O-saturated aqueous solution containing 5 mM TMP was irradiated with X-rays in the presence of 10 mM MNP and separated by reverse-phase HPLC in the ion-suppression mode. The arrow labeled d indicates the fraction corresponding to fraction d in Figure 1b. (c) Elution profile obtained from fraction d after purification once by reverse-phase HPLC, standing for 20 h at room temperature, and separation by reverse-phase HPLC in the ion-suppression mode. The arrow indicates the elution of unaltered thymine. The chromatogram was recorded by increasing the sensitivity by 160 times. All separations were carried out under the same conditions as in Figure 1b.

was observable. All fractions corresponding to fractions a–f in Figure 1b were immediately once again purified by HPLC under the same conditions. Each fraction was then injected into the HPLC column to detect the thymine base released from the separated spin adduct that was allowed to stand for an appropriate interval at room temperature. One example is shown in Figure 3c, in which the chromatographic profile obtained from the fraction corresponding to d is presented. In this case, the chromatogram was recorded by increasing the sensitivity by 160 times after the fraction was held for 20 h at room temperature. As is shown by the arrow in Figure 3c, the release of unaltered thymine base was observed. The amount of thymine base released increased with time. For fractions a and b, the liberation of unaltered thymine base was also observed, whereas no liberation of unaltered thymine base was observed in fractions c, e, and f.

Figure 4 represents the kinetics in the release of thymine base from fractions a, b, and d. A plot of log (fraction of the decreased amount of the spin adduct) vs time gave a straight line. From the slopes of the lines, the decay constants were calculated to be 0.0037, 0.0336, and 0.0113 h^{-1} for spin adducts a, b, and d, respectively. Since the initial concentrations of these adducts were similar, the order of stability of the spin

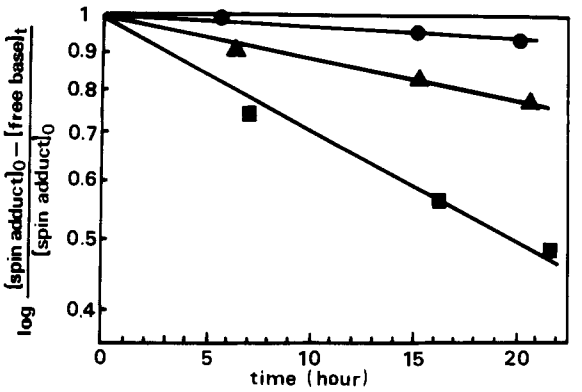

FIGURE 4: Plots of log $[[(\text{spin adduct})_0 - (\text{free base})_t]/(\text{spin adduct})_0]$ vs time (h), where $(\text{spin adduct})_0$ means the amount of the spin adduct observed in each fraction immediately after 100 μL of aqueous solution containing 5 mM TMP and 10 mM MNP was irradiated to a dose of 5.4 kGy of X-rays under N_2O, separated by HPLC, and purified once again by HPLC and $(\text{free base})_t$ means the amount of unaltered thymine base released from the spin adduct at t (h). (●) Spin adduct recovered in the fraction corresponding to a in Figure 1b. (▲) Spin adduct recovered in the fraction corresponding to d in Figure 1b. (■) Spin adduct recovered in the fraction corresponding to b in Figure 1b.

Table II: ESR Parameters of TMP Radicals and ddTMP Radicals Trapped by MNP[a]

fraction	radical trapped	hyperfine splitting constant (mT) a_N	a_{H^β}	a_{N^β}
	TMP Radicals			
a	C5′	1.50	0.56	
b	C1′	1.44		0.32
c	C5	1.58		
d	C4′	1.60		
e	C6	1.48		0.28
f	C5	1.58		
	ddTMP Radicals			
a	C5′	1.48	0.56	
a	C2′ or C3′	1.49	0.14	
b	C1′	1.44		0.30
c	C5	1.59		
d	C4′	1.62		
e	C6	1.50		0.25
f	C5	1.59		

[a] a_N denotes a primary ^{14}N splitting of the hyperfine structure. a_{H^β} and a_{N^β} denote secondary splitting by a β-hydrogen and β-nitrogen, respectively.

adducts was found to be a, d, and b. From this observation it could be concluded that the spin trapping undoubtedly occurred at the sugar moiety and also that the spin adducts were not very stable after prolonged standing.

Identification of the Spin Adducts at the Sugar Moiety with the Aid of ESR Spectroscopy. Identification of the spin-trapped radicals was made by analyzing the multiplicity and the magnitude of the hyperfine splittings of each ESR spectrum shown in Figure 2. The ESR spectrum of the spin adduct necessarily consists of a primary triplet hyperfine structure because of the interaction of a spin with a nuclear magnetic moment of nitrogen of the nitroxide group. The secondary hyperfine structure can help us to assign the chemical structure of the trapped radical. The hyperfine splittings of ESR spectra are summarized in Table II. The ESR spectrum of the spin adduct in fraction a shows a hyperfine structure consisting of a primary triplet of 1.50 mT and a secondary doublet of 0.56 mT. This secondary hyperfine structure could be analyzed on the basis of the interaction of a spin with a hydrogen at the β position (Riesz & Rustgi, 1979). The spin adduct at

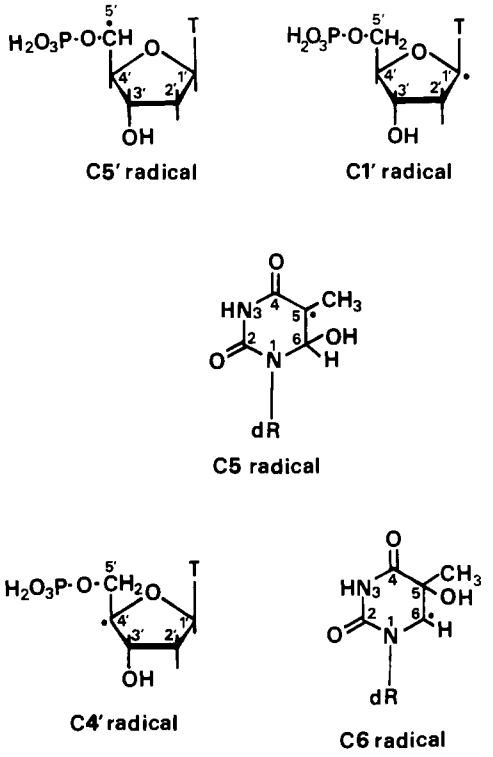

FIGURE 5: Untrapped forms of radicals isolated by HPLC and identified by ESR.

the C5′ position of the sugar was consistent with this spectrum. The untrapped form of the C5′ radical is shown in Figure 5.

As another candidate, the spin adduct between a hydrogen abstraction radical at the C2′ position and MNP was considered, but this possibility could be excluded by the fact that a quite similar spectrum was observed in uridine and 3′-UMP, which possessed an OH group at the C2′ position instead of an H group (Inanami et al., 1986, 1987), and because the spin adduct between the C2′ radical of 2′-chloro-2′-deoxyuridine and MNP gave a different ESR spectrum (Kuwabara et al., 1983).

The spin adduct recovered from peak b showed an ESR spectrum consisting of a primary triplet of 1.44 mT and a secondary triplet of 0.32 mT. The secondary triplet indicated that a spin of the adduct interacted with a nitrogen at the β position. The spin adduct between the C1′ radical of the sugar moiety and MNP is consistent with this spectrum. A similar result was obtained from 3′-UMP (Inanami et al., 1987). The untrapped form of the C1′ radical is shown in Figure 5.

The spin adduct from peak d showed an ESR spectrum consisting of only a primary triplet of 1.60 mT without further splittings, indicating that this spin adduct had no hydrogen or nitrogen atoms at the β position. A candidate for this spectrum is the spin adduct between the C4′ radical of the sugar moiety and MNP (shown as the untrapped form in Figure 5). Another candidate is the spin adduct between the C3′ radical of the sugar moiety and MNP. This possibility was, however, ruled out when ddTMP was used for the spin trapping experiment. This compound has two hydrogens at the C3′ position. The formation of a spin adduct between the C3′ radical and MNP would bring about an ESR spectrum consisting of a secondary doublet due to a β hydrogen. Thus, we can distinguish the spin adduct of the C4′ radical from that of the C3′ radical. The elution profile of the spin adducts between ddTMP radicals and MNP is presented in Figure 6a. The spin adducts were detected in fractions a–f. The ESR spectra obtained from fractions a–f corresponded to those

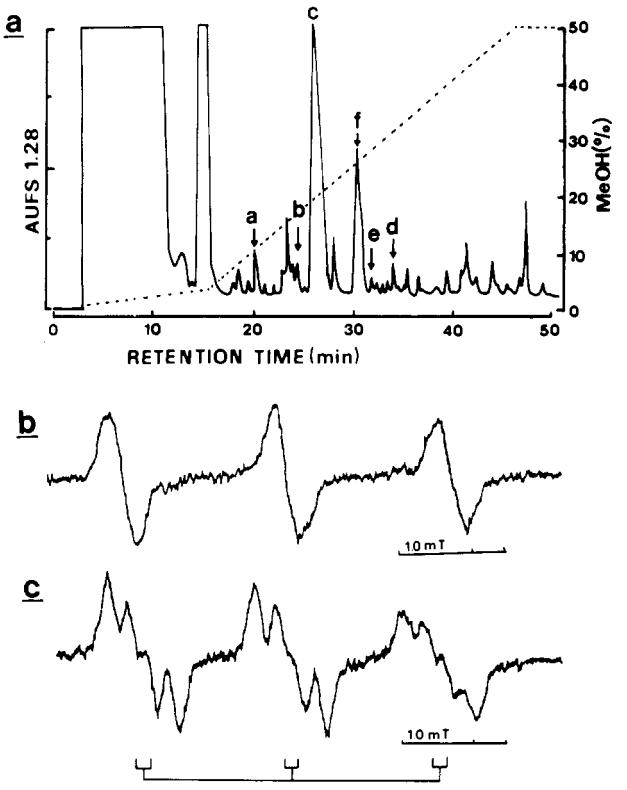

FIGURE 6: (a) Elution profile obtained after 1 mL of N_2O-saturated aqueous solution containing ddTMP and MNP was irradiated with X-rays and separated by reverse-phase HPLC in the ion-suppression mode. Separation conditions are the same as in the case of TMP. Each ESR spectrum obtained from fractions a–f corresponds to that obtained from fractions a–f in Figure 1b, respectively. (b) ESR spectrum of the spin adduct contained in fraction d. (c) ESR observation of the spin adducts contained in fraction a. The overlap of two ESR spectra was observed. The hyperfine structure of one spectrum is indicated under the spectrum.

obtained from fractions a–f in Figure 1, respectively. Figure 6b shows the ESR spectrum of a spin adduct belonging to fraction d. This spectrum consists of only a primary triplet hyperfine structure and has a somewhat broad line width compared to the spectrum shown in Figure 2d. Furthermore, this spin adduct eluted more slowly than the corresponding spin adduct of TMP did, as shown in Figure 1b. Nevertheless, this is attributable to the same spin adduct as that obtained from TMP, i.e., the spin adduct between the C4′ radical and MNP, because replacement of the OH group by a hydrogen at the C3′ position changes the chromatographic property of molecules and the broadening of line width can be explained by the interaction of a spin with four hydrogens at the γ position. The result using ddTMP indicated that the ESR spectrum in Figure 2d could be assigned to the spin adduct between the C4′ radical and MNP. In fraction a of Figure 6a, an ESR spectrum consisting of a secondary doublet of 0.14 mT was observed together with an ESR spectrum consisting of a secondary doublet of 0.56 mT that was assigned to the spin adduct between the C5′ radical and MNP (Figure 6c). Though the two ESR spectra could not be separated by HPLC, it was proved that each appeared from an individual spin adduct because the ESR spectrum consisting of a secondary doublet of 0.56 mT decayed more rapidly than the ESR spectrum consisting of a secondary doublet of 0.14 mT did. This spectrum was not observed in the case of TMP and therefore seems to be consistent with the spin adduct between the C3′ (or C2′) radical and MNP. The hyperfine splittings of ESR spectra for ddTMP are summarized together with those for TMP in Table II.

As described by Perkins et al. (1979) and Janzen (1971), trapping of a hydroxyalkyl radical like >Ċ–OH by MNP appears to form *tert*-butyl hydronitroxide (*t*-Bu–NHO•) rather than a spin adduct between the hydroxyalkyl radical and MNP. This *tert*-butyl hydronitoxide is very unstable and disappears rapidly. If the same argument is assumed to be applicable in the case of the C3′ radical of TMP, the observation of the spin adduct between the C3′ radical and MNP seems less likely, even though this radical is initially trapped by MNP.

Identification of the Spin Adducts at the Base Moiety with the Aid of ESR Spectroscopy. The UV absorbance spectra of the spin adducts in fractions c, e, and f in Figure 1 showed the absence of a chromophore at 260 nm (Figure 2). Furthermore, the adducts showed no release of unaltered bases. Thus, these adducts were regarded as those of the base radicals. The ESR spectra of fractions c and f consisted of only a primary triplet of 1.58 mT and were almost the same as each other. A quite similar spectrum was reported in our previous paper with dThd (Kuwabara et al., 1987), and another one was obtained in the present experiment with ddTMP (see Table II). In analogy with dThd, both spectra c and f are assigned to the spin adduct at the C5 position of the base moiety. The C5 radical is produced not only by OH addition to the C6 position of the 5,6 double bond but also by the cyclization reaction between the C6 position and the C3′ or C5′ radical, both of which are derived from hydrogen abstraction by OH radicals. The reaction between the C5′ radical and the C6 position is less likely because the C5′ radical was trapped by MNP as shown in Figure 2a. A spin trapping study was recently carried out on 2′-deoxycytidine 5′-monophosphate. In this case the ESR spectrum due to the spin adduct consistent with the C5 radical, which was induced by the attack of the C5′ radical on the C6 position to form C5′–C6 cyclization, was observed, while no ESR spectrum due to the spin adduct between the C5′ radical and MNP was recognized (unpublished results). This means that if the C5 radical is induced by the cyclization reaction between the C5′ radical and the 5,6 double bond of the base moiety, the spin adduct corresponding to the sugar radical is not detected. Since no ESR spectrum corresponding to the adduct between the C3′ radical and MNP was observed, the possibility of the adduct of the C5 radical, which is induced by the attack of the C3′ radical on the C6 position, cannot be excluded. However, analytic studies of OH-induced products showed no formation of C3′–C6 cyclo compounds in the case of dThd (von Sonntag, 1987). ESR signals obtained from chromatographic peaks c and f were relatively intense, and this was consistent with the fact that the majority of OH radicals (80%) reacted with the base moiety (Têoule & Cadet, 1978). Therefore, the assignment of ESR spectra belonging to peaks c and f to the C5 radical due to OH addition at the C6 position is reasonable.

It has been reported that the OH-induced C6 radical at the base moiety has a reducing property and can abstract a hydrogen from the sugar to form, for example, the C3′ radical (von Sonntag, 1987). Trapping of this radical by MNP also gives an ESR spectrum consisting of only a primary triplet and no maximum absorbance at 260 nm. However, as has already been discussed above, the trapping of the C3′ radical by MNP seems less likely. The untrapped form of the C5 radical is presented in Figure 5. One spin adduct was used to identify two spectra. This may be explained by assuming that this adduct has two structures. A similar observation was obtained in the case of 3′-UMP (Inanami et al., 1987). Several stereoisomers of the ring-saturated forms of thymine base in

γ-irradiated DNA have been reported (Breimer & Lindahl, 1985; Furlong et al., 1986; Teebor et al., 1987).

The ESR spectrum of fraction e consisted of a secondary triplet of 0.28 mT, indicating that a spin interacts with an α- or β-nitrogen. Since an examination of the spin adduct by UV absorbance spectrophotometry demonstrated the absence of maximum absorbance at 260 nm, this spectrum was assigned to the spin adduct of the C6 radical, though no hyperfine structure due to a hydrogen at the C6 position was observed in the ESR spectrum. As another candidate, the spin adduct between a nitrogen-centered radical (N1 or N3) and MNP was considered. In our previous paper with dThd (Kuwabara et al., 1987), we reported the observation of an ESR spectrum similar to that shown in Figure 2e. The application of another type of spin trapping reagent, α-(1-oxy-4-pyridyl)-*N*-*tert*-butylnitrone, clearly showed that no spin adducts between the nitrogen-centered radicals at the base moiety and the spin trapping reagent were produced in dThd. The same experiment was carried out in the present study, and no formation of spin adducts of nitrogen-centered radicals was found. The reason for the lack of hyperfine splitting due to the C6-hydrogen is considered to be as follows. The magnitude of hyperfine splitting due to hydrogen in the nitroxide spin adduct depends on the dihedral angle (θ) that the β-hydrogen makes with the p(π) orbital of the nitrogen of the nitroxide group, $a_{H^\beta} \sim \cos^2(\theta)$ (Heller & McConnell, 1960; Riesz & Rustgi, 1979). It is possible that minimum or zero splitting is observed when $\theta \simeq 90°$ sterically in the spin adduct between the C6 radical and MNP. The untrapped form of the C6 radical is shown in Figure 5. The C5 and C6 radicals are thought to be precursors of various oxidative conversions of the thymine base moiety.

It should be noted that the spin adduct of the hydrogen abstraction radical at the 5-methyl group of the base moiety was observed in both TMP and ddTMP but that it decayed rapidly. This base radical is also thought to be a precursor of various oxidative conversions of the thymine base moiety.

Discussion

Three radicals (C1′, C4′, and C5′) at the sugar moiety of TMP are formed as a result of hydrogen abstraction by OH radicals. These radicals have been inferred to be precursor radicals for end products which are induced in aqueous solutions of nucleotides, oligo- or polynucleotides, and isolated or intact cellular DNA by irradiation with ionizing radiation or antibiotic treatments (von Sonntag, 1984; Têoule, 1987; Teebor et al., 1988).

In 1980 von Sonntag reported that strand breaks with a formyl phosphate on the 3′-terminus and 5′-phosphate end group and with a formyl phosphate on the 3′-terminus and an altered sugar on the 5′-terminus accompanied by the release of unaltered bases and altered sugars result from the initial abstraction of a hydrogen at the C5′ position followed by O_2 addition. Antitumor antibiotic substances can also produce strand breaks of DNA. Povirk and Goldberg (1985) have inferred that, under aerobic conditions, thiol-activated neocarzinostatin abstracts a hydrogen from the C5′ of the sugar, resulting in thymine release as well as DNA strand breaks with nucleoside 5′-aldehyde at the 5′-end and a 3′-phosphate end group.

2′-Deoxyribonolactone has been considered to be an AP site formed through the C1′ radical (Isildar et al., 1981; Povirk & Goldberg, 1987). This product is an alkali-labile site that turns into a strand break upon alkali treatment but is known to be relatively resistant to cleavage by AP endonucleases (Povirk & Goldberg, 1987).

The characterization of C4′ radical mediated DNA damage has been done in an irradiated DNA solution as well as in antitumor drug treated DNA. It was reported by von Sonntag et al. (1981) that strand breaks with phosphate groups on both the 5′- and 3′-termini, which have gaps resulting from the loss of one nucleoside moiety, and strand breaks with an altered sugar on the 3′-terminus (or 5′-terminus) and a 5′-phosphate end group (or a 3′-phosphate end group), which involve the release of unaltered bases, are formed through the C4′ radical. Henner et al. (1983) and Schulte-Frohlinde and Bothe (1984) have reported that ionizing radiation causes destruction of the sugar moiety, resulting in a 5′-phosphate on one side of the gap and a 3′-(phosphoglycolate) on the other. This is thought to result from hydrogen abstraction at the C4′ position. Giloni et al. (1981) and Burger et al. (1986) have demonstrated that treatment of DNA with bleomycin in the presence of ferrous ions causes the formation of strand breaks in which a sugar fragment (glycolate) is linked to a 3′-phosphate group followed by the release of base propenal. The treatment of DNA with bleomycin resulted in the formation of an AP site (3,5-dihydro-4-oxopentanal residue) that was relatively resistant to AP endonucleolytic activity (Povirk & Goldberg, 1987). The C5′ radical is also thought to be an intermediate in the formation of this AP site.

In the present paper, evidence for the OH induction of sugar radicals in TMP was obtained by a method combining spin trapping and paired-ion HPLC. Much effort has been made to detect free-radical inductions at the sugar moiety of TMP resulting from both direct and indirect effects of ionizing radiation (Hüttermann et al., 1978). But only one paper, which reported the observation of an alkoxy sugar radical induced by direct absorption of ionizing energy with a single crystal of dThd at 4.2 K, is available (Box & Budzinski, 1975). The present experiment is the first evidence for the induction of carbon-centered radicals at the sugar moiety of TMP by ESR. Taking into consideration the fact that the TMP moiety in DNA is the most susceptible to modification by oxidative stress (Iida & Hayatsu, 1970) and ionizing radiation (Têoule & Cadet, 1978), these results will be useful for better understanding of the mechanisms of radiation-induced damage formation at the sugar moiety of DNA.

From studies of radiation-induced and antitumor drug induced end products using analytical techniques, many radical species at both base and sugar moieties have been proposed to explain the chemical processes which led to the end products (Teebor, 1988; Têoule, 1987; von Sonntag, 1987). The results of our previous and present spin trapping studies on DNA and RNA constitutents have given evidence for the presence of these presumed radicals (Hiraoka et al., 1989; Inanami et al., 1986, 1987; Kuwabara et al., 1983, 1987). We are now applying this method to the study of OH-induced free radicals in nucleosides and nucleotides corresponding to cytosine. As described by Teebor (1988) and Têoule (1987), there still remains uncertainty about the identification of chemical structures of OH-induced products in cytosine-related compounds. Exact identification of radical species that are induced in cytosine-related compounds will give significant information for the elucidation of OH-induced end products. In cytosine-related compounds, an interesting fact is that end products that are the same as those of uracil-related compounds have been identified. The saturation of the 5,6 double bond of the cytosine base by OH radicals has been believed to be a trigger for the deamination reaction to transform cytosine to uracil. Our spin trapping study of cytosine-related compounds clearly shows the time when the deamination reaction occurs after

initial OH attack on the double bond. Furthermore, this method demonstrates the formation of the C5 radical due to the cyclization reaction between the C5′ radical and the C6 position with subsequent deamination, though the OH-induced cyclo compound has never isolated (unpublished results). To elucidate the chemical processes which lead to OH-induced damage in DNA, its constituents have usually been employed as models. The spin trapping method presented here should be applied to further experiments with several DNA derivatives such as 5-halo-substituted pyrimidine nucleotides, with special emphasis on the elucidation of radio- and photosensitizing mechanisms. It will also be possible to apply this method to studies of the chemical processes induced by oxidative stress inside cells and chemical processes induced by vacuum UV light and synchrotron radiation from the electron storage ring in biologically significant molecules.

ACKNOWLEDGMENTS

We thank Drs. Tohru Ueda and Akira Matsuda, Faculty of Pharmaceutical Sciences, Hokkaido University, for the synthesis of 2′,3′-dideoxythymidine 5′-monophosphate.

Registry No. TMP, C5′ radical, 123438-18-6; TMP, C1′ radical, 123438-19-7; TMP, C5 radical, 123438-20-0; TMP, C4′ radical, 123438-21-1; TMP, C6 radical, 123438-22-2; TMP, 365-07-1; OH, 3352-57-6.

REFERENCES

Anbar, M., Bambenek, M., & Ross, A. B. (1973) *Natl. Stand. Ref. Data Ser. (U.S., Natl. Bur. Stand.) 43.*

Anbar, M., Farhataziz, & Ross, A. B. (1975) *Natl. Stand. Ref. Data Ser. (U.S., Natl. Bur. Stand.) 51.*

Box, H. C., & Budzinski, E. E. (1975) *J. Chem. Phys. 62*, 197–199.

Breimer, L. H., & Lindahl, T. (1985) *Biochemistry 24*, 4018–4022.

Burger, R. M., Projan, S. T., Horwitz, S. B., & Peisach, J. (1986) *J. Biol. Chem. 262*, 15955–15959.

Dorfman, L. M., & Adams, G. E. (1973) *Natl. Stand. Ref. Data Ser. (U.S., Natl. Bur. Stand.) 46.*

Furlong, E. A., Jorgensen, T. J., & Henner, W. D. (1986) *Biochemistry 25*, 4344–4349.

Giloni, L., Takeshita, M., Johnson, F., Iden, C., & Grollman, A. P. (1981) *J. Biol. Chem. 256*, 8608–8615.

Greenstock, C. L. (1978) *Abstracts of the 61st Canadian Chemical Congress*, OR-5, p 65.

Heller, C., & McConnell, H. M. (1960) *J. Chem. Phys. 32*, 1535–1539.

Henglein, A., Schnabel, W., & Wendenburg, J. (1969) *Einführung in die Strahlenchemie mit praktischen Anleitungen*, Verlag Chemie GmbH, Weinheim.

Henner, W. D., Rodriguetz, L. Q., Hecht, S. M., & Heseltine, W. A. (1983) *J. Biol. Chem. 258*, 711–713.

Hiraoka, W., Kuwabara, M., & Sato, F. (1989) *Int. J. Radiat. Biol. 55*, 51–58.

Hüttermann, J., Herak, J. N., & Westhof, E. (1978) in *Effects of Ionizing Radiation on DNA* (Hüttermann, J., Köhnlein, W., Téoule, R., & Berchinchamps, A. J., Eds.) pp 31–55, Springer-Verlag, New York, Heidelberg, and Berlin.

Iida, S., & Hayatsu, H. (1970) *Biochim. Biophys. Acta 213*, 1–13.

Inanami, O., Kuwabara, M., Endoh, D., & Sato, F. (1986) *Radiat. Res. 108*, 1–11.

Inanami, O., Kuwabara, M., & Sato, F. (1987) *Radiat. Res. 112*, 36–44.

Ip, Y. C., Ha, D., Morris, P. W., Puttemans, M. L., & Venton, D. L. (1985) *Anal. Biochem. 147*, 180–185.

Isildar, M., Schuchmann, M. N., Schulte-Frohlinde, D., & von Sonntag, C. (1981) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 40*, 347–354.

Janzen, E. G. (1971) *Acc. Chem. Res. 4*, 31–40.

Joshi, A., Rustgi, S., & Riesz, P. (1976) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 30*, 151–170.

Kominami, S., Rokushika, S., & Hatano, H. (1977) *Radiat. Res. 72*, 89–99.

Kuwabara, M. (1988) in *Abstracts of the International Symposium on Free-Radical- and Radiation-Induced Damage to DNA* (Schulte-Frohlinde, D., von Sonntag, C., & Hüttermann, J., Eds.) pp 68–69, Max-Planck-Institut für Strahlenchemie, Mülheim.

Kuwabara, M., Zhang, Z.-Y., & Yoshii, G. (1982) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 41* 241–259.

Kuwabara, M., Yoshii, G., & Itho, T. (1983) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 44*, 219–224.

Kuwabara, M., Inanami, O., Endoh, D., & Sato, F. (1987) *Biochemistry 26*, 2458–2465.

Lindahl, T. (1982) *Annu. Rev. Biochem. 51*, 66–87.

Perkins, M. J., Ward, P., & Horsfield, A. (1970) *J. Chem. Soc. B*, 395.

Povirk, L. F., & Goldberg, I. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3182–3186.

Povirk, L. F., & Goldberg, I. H. (1987) *Biochimie 69*, 815–823.

Riesz, P., & Rustgi, S. (1979) *Radiat. Phys. Chem. 13*, 21–40.

Schulte-Frohlinde, D., & Bothe, E. (1984) *Z. Naturforsch. 39C*, 315–319.

Teebor, G., & Frenkel, K. (1983) *Adv. Cancer Res. 38*, 23–59.

Teebor, G., & Boorstein, R. J., & Cadet, J. (1988) *Int. J. Radiat. Biol. 54*, 131–150.

Teebor, G., Cummings, A., Frenkel, K., Shaw, A., Voituriez, L., & Cadet, J. (1987) *Free Radical Res. Commun. 2*, 303–309.

Tener, G. M. (1961) *J. Am. Chem. Soc. 83*, 159–168.

Téoule, R. (1987) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 51*, 573–589.

Téoule, R., & Cadet, J. (1978) in *Effects of Ionizing Radiation on DNA* (Hüttermann, J., Köhnlein, W., Téoule, R., & Berchinchamps, A. J., Eds.) pp 171–203, Springer-Verlag, New York, Heidelberg, and Berlin.

von Sonntag, C. (1980) *Adv. Carbohydr. Chem. Biochem. 37*, 7–77.

von Sonntag, C. (1984) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 46*, 507–519.

von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, Taylor & Francis, London.

von Sonntag, C., & Schulte-Frohlinde, D. (1978) in *Effects of Ionizing Radiation on DNA* (Hüttermann, J., Köhnlein, W., Téoule, R., & Berchinchamps, A. J., Eds.) pp 204–235, Springer-Verlag, New York, Heidelberg, and Berlin.

von Sonntag, C., Hagen, U., Schön-Bopp, A., & Schulte-Frohlinde, D. (1981) *Adv. Radiat. Biol. 9*, 109–142.