Formation of an 8-Hydroxyguanine Moiety in Deoxyribonucleic Acid on γ-Irradiation in Aqueous Solution†

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ABSTRACT: Isolation and characterization of a novel radiation-induced product, i.e., the 8-hydroxyguanine residue, produced in deoxyribonucleic acid (DNA), 2′-deoxyguanosine, and 2′-deoxyguanosine 5′-monophosphate by γ-irradiation in aqueous solution, are described. For this purpose, γ-irradiated DNA was first hydrolyzed with a mixture of four enzymes, i.e., DNase I, spleen and snake venom exonucleases, and alkaline phosphatase. Analysis of the resulting mixture by capillary gas chromatography–mass spectrometry after trimethylsilylation revealed the presence of a product, which was identified as 8-hydroxy-2′-deoxyguanosine on the basis of the typical fragment ions of its trimethylsilyl (Me_3Si) derivative. This product was then isolated by using reversed-phase high-performance liquid chromatography. The UV and proton nuclear magnetic resonance spectra taken from the isolated product confirmed the structure suggested by the mass spectrum of its Me_3Si derivative. In addition, the accurate molecular mass of the Me_3Si derivative of the isolated product was determined by MS. The obtained value agreed with the theoretical molecular mass within 1 millimass unit. The yield of 8-hydroxyguanine was also measured. Its mechanism of formation is believed to involve OH radical addition to the C-8 position of guanine followed by oxidation of the radical adduct.

Deoxyribonucleic acid (DNA)[1] is considered to be the principal target for ionizing radiation-induced damage in living cells (Painter, 1980). Ionizing radiation creates a number of lesions in DNA such as modified bases, base-free sites, and single- and double-strand breaks (Ward, 1975); cross-linking between DNA and proteins is another type of damage caused by ionizing radiation (Mee & Adelstein, 1979). Lesions produced in DNA, however, are subject to repair processes (Friedberg, 1984) and, unless repaired, may have detrimental biological consequences (Painter, 1980). Knowledge of the chemical nature of radiation-induced alterations in DNA is necessary, therefore, for an understanding of their biological consequences and repairability.

The effects of ionizing radiation on DNA and its constituents have been studied quite extensively [for reviews, see Teoule & Cadet (1978) and Scholes (1983)]. The radiation chemistry of the purine bases of DNA, however, is not as well understood as that of pyrimidine bases. Several radiation-induced products of purines have been identified. 4,6-Diamino-5-formamidopyrimidine and 8-hydroxyadenine (8-OH-Ade) were found in irradiated aqueous solutions of adenine (Ade) and adenosine (Ado) (Hems, 1960; Conlay, 1963) as well as of DNA (Bonicel et al., 1980). The formamidopyrimidine derivative of guanine (Gua), i.e., 2,4-diamino-5-formamido-6-hydroxypyrimidine, has also been observed in irradiated aqueous solutions of guanosine (Guo) and guanylic acid (Hems, 1958) as well as of DNA (Hems, 1960). In addition, a number of radiation-induced base and sugar products of 2′-deoxyguanosine (dGuo) have been described (Cadet & Berger, 1985; Cadet et al., 1983). However, the formation of the 8-hydroxyguanine (8-OH-Gua) moiety in DNA, or in Gua nucleosides and nucleotides, irradiated in aqueous solutions has not been reported.

This paper describes the identification of an 8-OH-Gua moiety in DNA, dGuo, and 2′-deoxyguanosine 5′-monophosphate (5′-dGMP) γ-irradiated in aqueous solutions. High-performance liquid chromatography (HPLC), capillary gas chromatography–mass spectrometry (GC-MS), UV spectroscopy, nuclear magnetic resonance spectroscopy (NMR), and accurate mass determination by MS were used for this purpose.

Materials and Methods[2]

Materials. Calf thymus DNA, dGuo, 5′-dGMP, uracil, and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma. Acetonitrile was obtained from Pierce. Water purified through a Millipore system was used for all purposes. All enzymes used were purchased from Boehringer Mannheim.

Irradiations. Aqueous solutions of DNA (0.25 μg/mL), dGuo, and 5′-dGMP (both 1 mM) were saturated with N_2O or N_2O/O_2 (1:1) for 30 min and irradiated in a ^{60}Co γ source (dose range 91–364 J/kg; dose rate 91 J/kg·min) (1 J/kg = 1 Gy = 100 rad). The samples were then lyophilized.

High-Performance Liquid Chromatography. Separations were carried out on a Hewlett-Packard Model 1090 microprocessor-controlled liquid chromatograph equipped with a Model HP 1040A high-speed spectrophotometric detector and a 10 × 0.2 cm Hypersil ODS microbore reversed-phase column (particle size 5 μm; Hewlett-Packard). For semipreparative separations, a Supelcosil LC-8-DB column (25 × 1 cm; particle

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[1] Abbreviations: DNA, deoxyribonucleic acid; 8-OH-Ade, 8-hydroxyadenine; Ade, adenine; Ado, adenosine; Gua, guanine; Guo, guanosine; dGuo, 2′-deoxyguanosine; dAdo, 2′-deoxyadenosine; 8-OH-Gua, 8-hydroxyguanine; 8-OH-dGuo, 8-hydroxy-2′-deoxyguanosine; 5′-dGMP, 2′-deoxyguanosine 5′-monophosphate; HPLC, high-performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance; UV, ultraviolet; J, joule; BSTFA, bis(trimethylsilyl)trifluoroacetamide; DNase I, deoxyribonuclease I; kPa, kilopascal; eV, electron volt; B, base; Me_3Si (TMS in figures), trimethylsilyl; Me_3SiOH, trimethylsilanol; $M^{+\cdot}$, molecular ion; S, sugar; FID, flame ionization detector; RMRF, relative molar response factor; δ, proton chemical shift; amu, atomic mass unit; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] Mention of commercial products does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the products identified are necessarily the best available for the purpose.

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size 5 μm; Supelco) was used.

Enzymatic Hydrolysis. One milligram of DNA samples was dissolved in 1 mL of 40 mM Tris-HCl buffer (pH 8.5) containing 10 mM $MgCl_2$. This solution was incubated with DNase I (200 units), spleen exonuclease (0.01 unit), snake venom exonuclease (0.5 unit), and alkaline phosphatase (10 units) for 2 h at 37 °C. Then, an aliquot of this solution was injected into the liquid chromatograph. Another aliquot was lyophilized and used for GC–MS analysis.

Hydrolysis with Formic Acid. In order to remove the bases from the sugar moieties of DNA, dGuo, or 5′-dGMP, 1 mg of each sample was treated with 1 mL of concentrated formic acid (88%) in evacuated and sealed tubes at 150 °C for 30 min. After hydrolysis, samples were dried in a desiccator under vacuum prior to trimethylsilylation.

Trimethylsilylation. Samples were trimethylsilylated in PTFE-capped hypovials (Pierce) with 0.2 mL of a mixture of BSTFA and acetonitrile (1:1) by heating for 15 min at 130 °C.

Gas Chromatography–Mass Spectrometry. A Hewlett-Packard Model 5880A microprocessor-controlled gas chromatograph equipped with a flame ionization detector (FID) was used. The injection port and detector were both maintained at 250 °C. Separations were carried out on a fused silica capillary column (12 m, 0.2 mm i.d.) coated with cross-linked SE-54 (5% phenylmethyl silicone; film thickness 0.11 μm) (Hewlett-Packard). Helium was used as the carrier gas at an inlet pressure of 100 kPa. the split ratio was 20:1. Mass spectra were taken at 70 eV on a Hewlett-Packard Model 5970A mass selective detector interfaced to the above gas chromatograph. The temperature of the ion source was ca. 200 °C.

Nuclear Magnetic Resonance Spectroscopy. ^1H NMR spectra were recorded in the pulse, Fourier-transform mode at 400 MHz using a Bruker Instruments Model WM-400 spectrometer with a 45° pulse (20 μs), a 16 384-point data set, and a spectral width of 5 kHz. For this purpose, approximately 1 mg of the product isolated by HPLC was dissolved in 0.6 mL of deuterium oxide containing sodium 4,4-dimethyl-4-silapentanoate-*2,2,3,3-d*$_4$ as the reference compound. For comparison, the ^1H NMR spectrum of dGuo was also recorded in the same manner.

Accurate Mass Determination. A VG Analytical Model 70E-HF mass spectrometer was used for accurate mass determination at a resolution of 3000. The scans were taken in a linear up mode with the field control at 10 s/scan. The data were acquired in continuum mode as single scans with perfluorokerosine being let in as the reference. The ion source temperature was 210 °C.

Results

All samples were analyzed by GC–MS. Figures 1 and 2 show the total ion chromatograms obtained from trimethylsilylated enzymatic digests of γ-irradiated and nonirradiated DNA samples, respectively. In order to find out whether 8-hydroxy-2′-deoxyguanosine (8-OH-dGuo) was present in these samples, the "base + H" (B + 1) ion (*m/z* 383), which is characteristic of trimethylsilyl (Me_3Si) derivatives of nucleosides (McCloskey et al., 1968; McCloskey, 1974; Pang et al., 1982), was monitored simultaneously. Peaks 1 and 3 in Figures 1 and 2 correspond to Me_3Si derivatives of 2′-deoxyadenosine (dAdo) and dGuo, respectively. The upper portion of Figure 1 clearly shows the presence of the *m/z* 383 ion in the mass spectrum of the compound represented by peak 4 in Figure 1. As Figure 2 clearly shows, this compound was not present in nonirradiated DNA samples. The mass spectrum taken from peak 4 in Figure 1 is illustrated in Figure 3. The interpretation was based on the well-known fragmentation patterns of the Me_3Si derivatives of nucleosides (McCloskey et al., 1968; McCloskey, 1974; Pang et al., 1982). A molecular ion ($M^{+\cdot}$) and a characteristic $(M - CH_3)^+$ ion were observed at *m/z* 643 and 628, respectively. The ion at *m/z* 538 corresponds to the loss of trimethylsilanol (Me_3SiOH; 90 amu) from *m/z* 628. These ions clearly indicate the addition of an OH (or OMe_3Si) group to the dGuo molecule, which shows an $M^{+\cdot}$ ion at *m/z* 555 in the mass spectrum of its Me_3Si derivative (McCloskey, 1974). The most intense

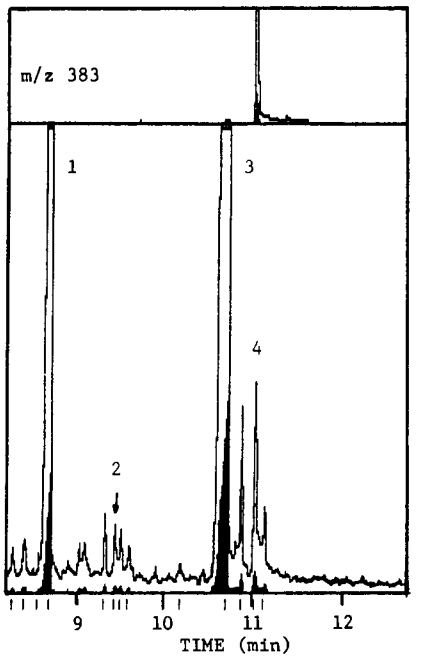


FIGURE 1: Total ion chromatogram obtained from a trimethylsilylated enzymatic hydrolysate of γ-irradiated DNA (dose 273 J/kg). Column, fused silica capillary coated with cross-linked SE-54 (12 m × 0.2 mm i.d.), programmed from 190 to 250 °C at 7 °C/min after 3 min at 190 °C. Column head pressure 100 kPa. The upper portion shows the single ion (*m/z* 383) chromatogram recorded simultaneously.

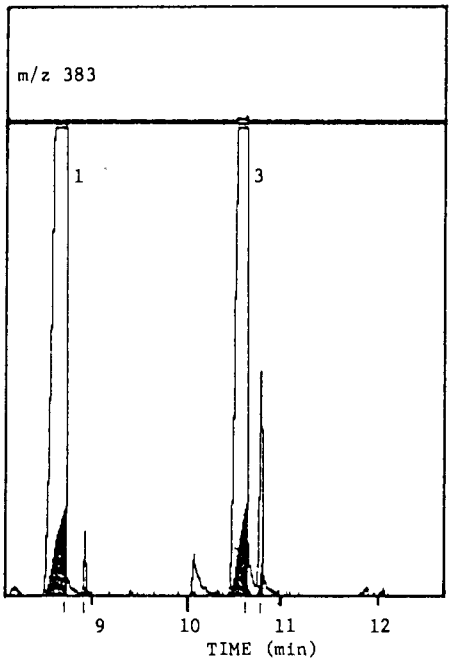


FIGURE 2: Total ion chromatogram obtained from a trimethylsilylated enzymatic hydrolysate of nonirradiated DNA. Other details as in Figure 1.

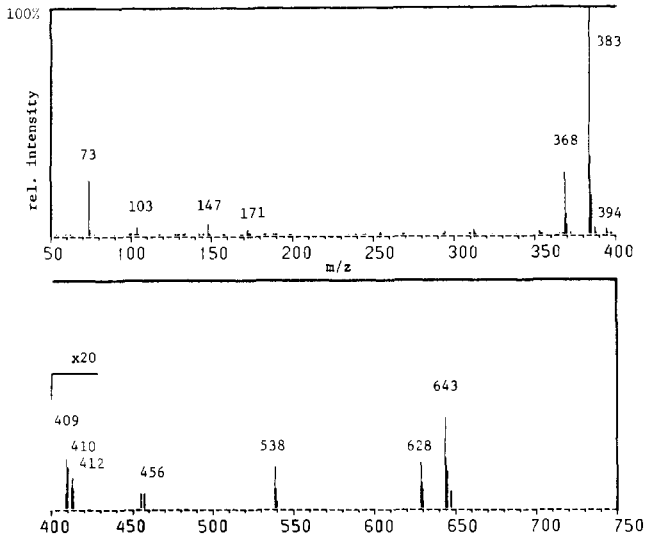
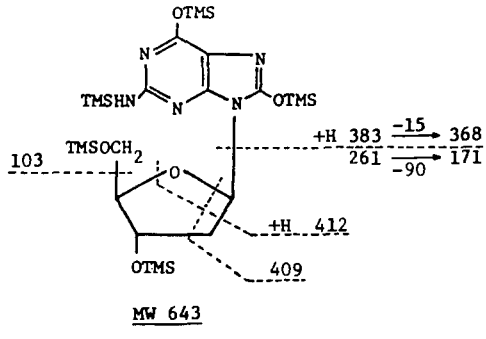


FIGURE 3: Mass spectrum taken from peak 4 in Figure 1.

ion at m/z 383 in Figure 3 corresponds to the B + 1 ion and the second most intense ion at m/z 368 to the "base + H – CH_3" (B – 14) ion (Pang et al., 1982). When compared with the corresponding ions in the mass spectrum of MeSi–dGuo (McCloskey, 1974), these ions clearly show that the additional OMe_3Si group is located on the purine ring and not on the sugar moiety. It should be pointed out that the Me_3Si derivative of Guo has the same molecular ion (m/z 643) as that of 8-OH-dGuo. However, this compound exhibits a mass spectrum (McCloskey et al., 1968; McCloskey, 1974) quite different in terms of fragment ions from that in Figure 3 and had a different retention time from peak 4 under the GC conditions in Figure 1. Furthermore, Guo was not found in nonirradiated DNA. According to Pang et al. (1982), there exists a strong correlation in purines between the abundance of the B + 1 ion and the electronegativity of substituents at the C-8 atom of the purine ring. Stabilization through electron-donating substituents such as Br, OH, or $NHCH_3$ at C-8 causes an increase in the relative abundance of the B + 1 ion. In the mass spectrum shown in Figure 3, the B + 1 ion appears as the base peak and the B – 14 ion as the second most abundant ion (27% relative intensity), whereas the latter ion in the mass spectra of Me_3Si–dGuo and –Guo is more intense than the B + 1 ion (McCloskey et al., 1968; McCloskey, 1974). The same effect was also observed when the mass spectra of Me_3Si–Ado and –8-OH-Ado were compared (McCloskey, 1974; Pang et al., 1982). These facts strongly suggest that the OH group is located at the C-8 of the purine ring of the product with the mass spectrum shown in Figure 3. This radiation-induced product of DNA was therefore attributed to 8-OH-dGuo. The following is its Me_3Si derivative along with the typical fragmentation patterns:



Ions containing the base plus portions of the sugar (S) moiety

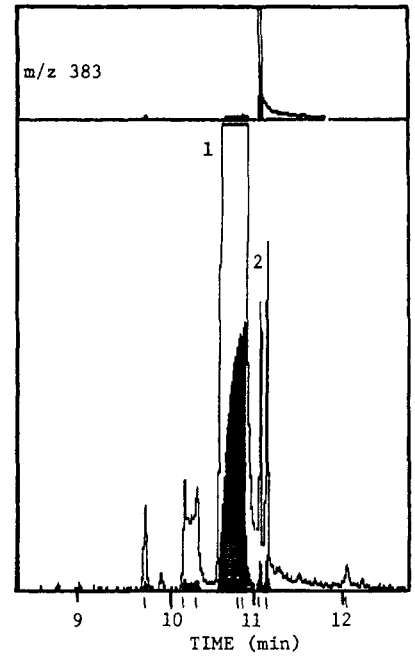


FIGURE 4: Total ion chromatogram obtained from a trimethylsilylated sample of irradiated 2′-deoxyguanosine (dose 273 J/kg). Other details as in Figure 1.

(Pang et al., 1982) appear at m/z 409 (base–$C^{1'}H$–$C^{2'}H_2$), 410 (m/z 409 + H), and 412 (base–$C^{1'}H$=O + H). The ion at m/z 456 corresponds to addition of HMe_3Si to the base (B + 74 ion). The loss of Me_3SiOH from the sugar moiety gives rise to the m/z 171 ion (S – 90). The ion at m/z 103 usually occurs in the mass spectra of nucleosides (Pang et al., 1982). The m/z 73 and 147 ions are commonly observed with Me_3Si derivatives and serve no diagnostic purpose. Peak 2 in Figure 1 might correspond to the Me_3Si derivative of 8-hydroxy-2′-deoxyadenosine, a product previously observed in γ-irradiated aqueous solutions of DNA (Bonicel et al., 1980). Peaks not numbered in Figures 1 and 2 were not defined.

GC–MS analysis also showed the presence of 8-OH-dGuo in γ-irradiated samples of dGuo and 5′-dGMP. A total ion chromatogram obtained from a sample of trimethylsilylated γ-irradiated dGuo is given in Figure 4, where peak 1 represents Me_3Si–dGuo. The presence of the m/z 383 ion is clearly seen in peak 2. The mass spectrum taken from this peak was identical with that in Figure 3. Irradiated 5′-dGMP gave an essentially identical chromatogram after removal of the 5′-terminal phosphate group by alkaline phosphatase followed by trimethylsilylation. Nonirradiated samples of dGuo and 5′-dGMP did not show the presence of 8-OH-dGuo or Guo.

All samples were also analyzed by GC–MS following hydrolysis with formic acid. Figures 5 and 6 show portions of the total ion chromatograms obtained from the trimethylsilylated formic acid hydrolysates of γ-irradiated and nonirradiated DNA samples, respectively. The $M^{+\cdot}$ ion (m/z 455) of the Me_3Si derivative of 8-OH-Gua expected from hydrolysis of 8-OH-dGuo was monitored simultaneously. The presence of this ion was observed in mass spectrum of the compound represented by peak 8 in Figure 5. Nonirradiated DNA samples did not contain this compound, as can be seen in Figure 6. The mass spectrum taken from peak 8 in Figure 5 is illustrated in Figure 7 and shows intense $(M–CH_3)^+$ and $M^{+\cdot}$ ions at m/z 440 (base peak) and 455 (93% relative intensity), respectively, reflecting the aromatic character of this molecule (White et al., 1972). This spectrum was attributed to the Me_3Si derivative of 8-OH-Gua, which arises from the

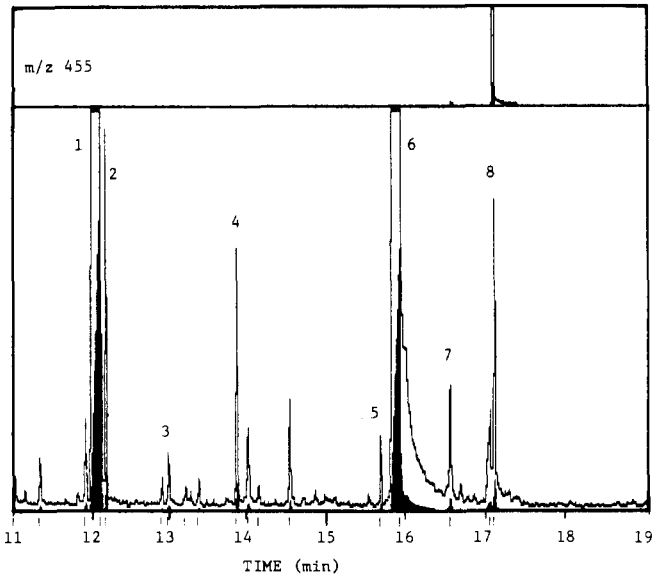


FIGURE 5: Total ion chromatogram obtained from a trimethylsilylated formic acid hydrolysate of γ-irradiated DNA (dose 273 J/kg). Column as in Figure 1. Temperature program, from 100 to 250 °C at 7 °C/min after 3 min at 100 °C. The upper portion shows the single ion (m/z 455) chromatogram recorded simultaneously.

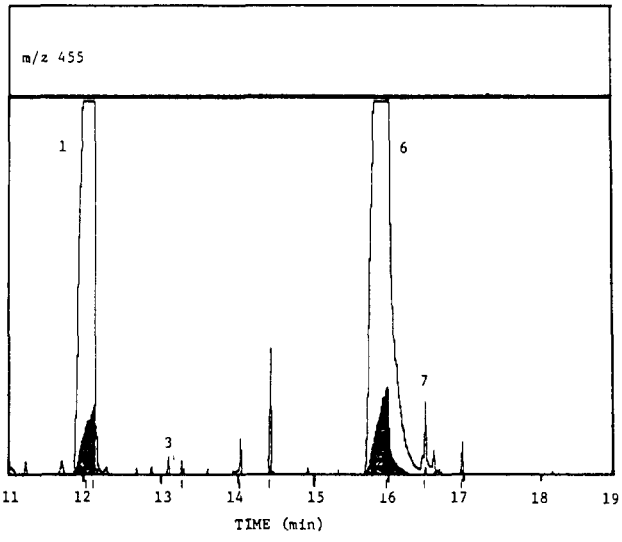


FIGURE 6: Total ion chromatogram obtained from a trimethylsilylated formic acid hydrolysate of nonirradiated DNA. Other details as in Figure 5.

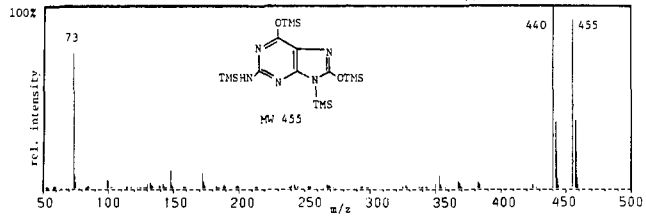


FIGURE 7: Mass spectrum taken from peak 8 in Figure 5.

acid hydrolysis of 8-OH-dGuo. Peaks 1 and 6 in Figures 5 and 6 represent the Me_3Si derivatives of Ade and Gua, respectively. Peaks 3 and 7 also correspond to the Me_3Si derivatives of Ade and Gua, respectively, but with an additional Me_3Si group attached to their amino groups, as revealed by their mass spectra. Peaks 2, 4, and 5 in Figure 5 represent Me_3Si derivatives of 4,6-diamino-5-formamidopyrimidine, 8-OH-Ade, and 2,4-diamino-5-formamido-6-hydroxypyrimidine, respectively, which have been identified previously in γ-irradiated DNA (see introduction). The absence of these

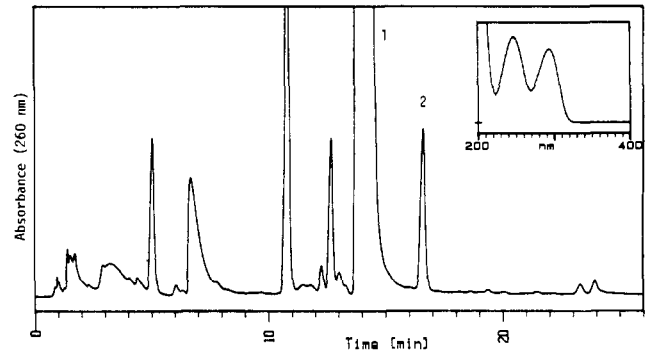


FIGURE 8: Liquid chromatogram obtained from γ-irradiated 2'-deoxyguanosine (dose 273 J/kg). Column, microbore Hypersil ODS (10 × 0.2 cm; particle size 5 μm). Flow rate 0.2 mL/min. Eluent: A, water; B, acetonitrile; gradient elution with a rate of 0.2% B/min. Temperature, ambient. Insert shows the UV spectrum taken from peak 2.

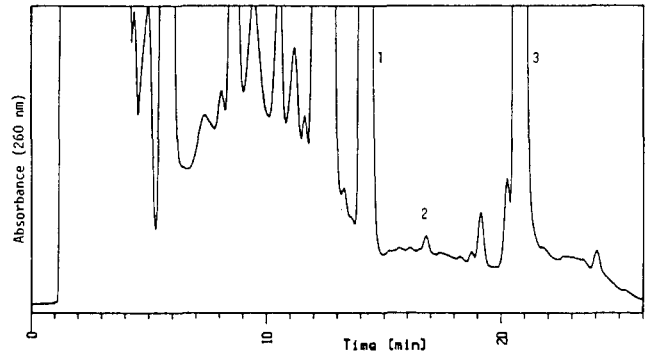


FIGURE 9: Liquid chromatogram obtained from an enzymatic hydrolysate of γ-irradiated DNA (dose 273 Gy). Column details as in Figure 8.

products in nonirradiated DNA samples is clearly seen in Figure 6. Peaks not numbered in both chromatograms were not defined. The trimethylsilylated formic acid hydrolysates of dGuo and 5'-dGMP also showed the presence of 8-OH-Gua on GC-MS analysis.

Irradiated samples were also analyzed by reversed-phase HPLC. A chromatogram obtained from γ-irradiated dGuo is shown in Figure 8. The insert illustrates the UV spectrum taken from peak 2 (the HPLC instrument used is capable of taking UV spectra during a run). UV spectra acquired on the upslope, apex, and downslope of this peak were identical, indicating its homogeneity. The corresponding product was collected by using a semipreparative HPLC column, and GC–MS analysis of its Me_3Si derivative gave a single peak with a mass spectrum identical with that shown in Figure 3. Furthermore, the collected product was hydrolyzed with formic acid, and subsequent HPLC analysis showed the presence of a single peak with a UV spectrum identical with that in Figure 8. It should be noted that this UV spectrum with two absorption maxima at 245 and 292 nm is the same as that previously published for synthesized 8-OH-Gua (Cavalieri & Bendich, 1950). Moreover, the GC–MS analysis of an aliquot of the formic acid hydrolysate revealed the presence of 2'-deoxyribose and 8-OH-Gua. On the basis of these data, the radiation-induced product represented by peak 2 in Figure 8 was attributed to 8-OH-dGuo. Peak 1 corresponds to dGuo. Other peaks in Figure 6 were not defined and may correspond to products previously observed (see introduction). The HPLC analysis of the enzymatic digest of irradiated DNA is shown in Figure 9. Peak 2 in this chromatogram had the same capacity factor and UV and mass spectra as peak 2 in Figure 8. It was therefore assigned to 8-OH-dGuo. Peaks 1 and 3

in Figure 9 represent dGuo and dAdo, respectively.

In accord with the structural evidence from GC–MS and UV spectroscopy, the ^1H NMR spectrum of the product isolated by HPLC (peaks 2 in Figures 8 and 9) chemical shifts (δ) 2.276 and 3.020 (2 H, C-2′), 3.802 and 3.868 (2 H, C-5′), 4.015 (H, C-4′), 4.569 (H, C-3′), and 6.245 (H, C-1′); δ of dGuo, 2.516 and 2.792 (2 H, C-2′), 3.760 and 3.823 (2 H, C-5′), 4.132 (H, C-4′), 4.624 (H, C-3′), 6.303 (H, C-1′), and 7.986 (H, C-8)]. Furthermore, the accurate molecular mass of the Me_3Si derivative of this product was determined by MS as 643.2903. This value agrees with the theoretical molecular mass (643.2893) for the composition $C_{25}H_{53}N_5O_5Si_5$ within 1 millimass unit.

The yield of 8-hydroxyguanine was determined by GC with uracil as an internal standard. Irradiated samples containing a certain amount of uracil were lyophilized and then subjected to hydrolysis with formic acid. This was followed by trimethylsilylation and GC analysis. Since there was no authentic material available, the relative molar response factor (RMRF) of Me_3Si–8-OH-Gua for the flame ionization detector (FID) was assumed to be equal to that of Me_3Si–Gua vs. Me_3Si–uracil based on the method for calculation of the RMRF's of Me_3Si derivatives for FID (Ackman, 1964; Verhaar & de Vilt, 1964). This was also supported by the fact that the RMRF of Me_3Si–Ade was found to be equal to that of Me_3Si–Gua vs. Me_3Si–uracil (Ade differs from Gua by an OH group, like Gua from 8-OH-Gua).

For determination of the RMRF's, certain amounts of uracil, dAdo, and dGuo solutions were mixed, lyophilized, and then subjected to formic acid hydrolysis. GC analysis after trimethylsilylation yielded an RMRF of 1.0 for both Me_3Si–Ade and Me_3Si–Gua vs. Me_3Si–uracil. In all instances, 8-OH-Gua gave linear dose–yield plots between the doses 91 and 364 J/kg. The G value (number of molecules formed per 100 eV of absorbed radiation energy) of 8-OH-Gua amounted to 0.17 and 0.55 for N_2O-saturated DNA and dGuo (or 5′-dGMP) samples, respectively. In the presence of oxygen, these values were 0.30 and 0.40, respectively.

Discussion

Radiation-generated OH radicals react with both the heterocyclic bases and the sugar moiety of DNA, but predominantly with the bases [for a review, see Scholes (1978)]. Reactions of OH radicals with DNA bases are characterized by predominant addition to the double bonds of these molecules. In the case of pyrimidines, the major site of attack is the 5,6-double bond; an abstraction of the H atom from the methyl group of thymine also occurs (Fujita & Steenken, 1981; Hazra & Steenken, 1983). Mechanistic details of OH radical reeactions with the purines of DNA certainly are not understood as well as those for the pyrimidines. The radiation-induced products of Ade (see introduction) could be accounted for by the addition of the OH radical to the C-8 position followed by the reduction or oxidation of the radical formed (van Hemmen & Bleichrodt, 1971). The radiation chemistry of Gua is even more controversial than that of Ade.

A recent pulse radiolysis study has suggested that the interaction of OH radical with dGuo and 5′-dGMP proceeds mainly by addition to the C-4, C-5, and C-8 positions of the Gua moiety to yield three radicals with different redox properties (O'Neill, 1983). Earlier studies proposed the formation of the radical cation upon OH radical attack at dGuo and 5′-dGMP (Willson et al., 1974). In a recent review article (Cadet & Berger, 1985), hydroxylation of the C-8 position of the purine moiety in dGuo or 5′-dGMP (or in DNA) was ruled out on the basis of theoretical calculations.

The formation of the 8-OH-Gua moiety in dGuo, 5′-dGMP, and DNA can be explained, similar to OH radical induced formation of 8-OH-Ade moiety in dAdo and DNA (Hems, 1960; Conlay, 1963; van Hemmen & Bleichrodt, 1971; Scholes 1983), by initial addition of the OH radical to the C-8 position of the purine moiety. The resulting OH adduct with a radical center at the N-7 position could then lead to 8-OH-Gua on oxidation. An alternative mechanism would be the formation of a glycol-type product, with an OH group both at C-8 and N-7, followed by dehydration to give 8-OH-Gua, as suggested previously for the formation of 8-OH-Ade (Conlay, 1963; van Hemmen & Bleichrodt, 1971).

Conclusions

The reactions of radiation-generated OH radicals with DNA, dGuo, and 5′-dGMP in aqueous solutions in the presence and absence of oxygen have been shown to lead to the formation of an 8-OH-Gua moiety. This product was isolated and characterized by using capillary GC–MS, HPLC, UV spectroscopy, ^1H NMR spectroscopy, and accurate mass determination. Its formation in DNA, dGuo, or 5′-dGMP is believed to result from the OH radical addition to the C-8 position of the guanine moiety followed by oxidation of the radical adduct formed.

It is hoped that the findings presented in the present work will help to resolve the controversial problems associated with the radiation chemistry of guanine.

Acknowledgments

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Registry No. 8-OH-Gua, 5614-64-2; dGuo, 961-07-9; 5′-dGMP, 902-04-5; 8-OH-Gua (Me_3Si derivative), 96964-33-9.

References

Ackman, R. G. (1964) *J. Gas Chromatogr. 2*, 173–179.
Bonicel, A., Mariaggi, N., Hughes, E., & Teoule, R. (1980) *Radiat. Res. 83*, 19–26.
Cadet, J., & Berger, M. (1985) *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 47*, 127–143.
Cadet, J., Voituriez, L., & Berger, M. (1983) *J. Chromatogr. 259*, 111–119.
Cavelieri, L. F., & Bendich, A. (1950) *J. Am. Chem. Soc. 72*, 2587–2594.
Conlay, J. J. (1963) *Nature (London) 197*, 555–557.
Friedberg, E. C. (1984) *DNA Repair*, W. A. Freeman, New York.
Fujita, S., & Steenken, S. (1981) *J. Am. Chem. Soc. 103*, 2540–2545.
Hazra, D. K., & Steenken, S. (1983) *J. Am. Chem. Soc. 105*, 4380–4386.
Hems, G. (1958) *Nature (London) 181*, 1721–1722.
Hems, G. (1960) *Nature (London) 186*, 710–712.
McCloskey, J. A. (1974) in *Basic Principles in Nucleic Acid Chemistry* (TS'o, P.O.P., Ed.) pp 209–309, Academic Press, New York.
McCloskey, J. A., Lawson, A. M., Tsuboyama, D., Krueger, P. M., & Stillwell, R. N. (1968) *J. Am. Chem. Soc. 90*, 4182–4184.
Mee, L. K., & Adelstein, S. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2194–2198.
O'Neill, P. (1983) *Radiat. Res. 96*, 198–210.

Painter, R. B. (1980) in *Radiation Biology in Cancer Research* (Meyn, R. E., & Withers, H. R., Eds.) pp 59–68, Raven Press, New York.
Pang, H., Schram, K. H., Smith, D. L., Gupta, S. P., Townsend, L. B., & McCloskey, J. A. (1982) *J. Org. Chem. 47*, 3932.
Scholes, G. (1978) in *Effects of Ionizing Radiation on DNA* (Hütterman, J. Köhnlein, W., & Teoule, R., Eds.) pp 153–170, Springer-Verlag, Berlin.
Scholes, G. (1983) *Br. J. Radiol. 56*, 221–231.
Teoule, R., & Cadet, J. (1978) in *Effects* of *Ionizing Radiation on DNA* (Hütterman, J., Köhnlein, W., & Teoule, R., Eds.) pp 171–203, Springer-Verlag, Berlin.
van Hemmen, J. J., & Bleichrodt, J. F. (1971) *Radiat. Res. 46*, 444–456.
Verhaar, L. A., & de Vilt, H. G. J. (1969) *J. Chromatogr. 41*, 168–179.
Ward, J. F. (1975) *Adv. Radiat. Biol. 5*, 181–239.
White, E., Krueger, P. M., & McCloskey, J. A. (1972) *J. Org. Chem. 37*, 430–438.
Willson, R. L., Wardman, P., & Asmus, K. D. (1974) *Nature (London) 252*, 323–324.

Kinetics and Mechanism of the Refolding of Denatured Ribonuclease A†

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ABSTRACT: On the basis of two experimental observations, it is established that the refolding mechanism of ribonuclease A (RNase A) is independent of the nature of the denaturant used [urea or guanidine hydrochloride (Gdn·HCl)]. First, by use of a double-jump technique, it is demonstrated that a similar nativelike intermediate exists on the major slow-folding pathway of both urea- and Gdn·HCl-denatured RNase A. Second, from the temperature dependence of the slow-refolding kinetics, it is shown that the activation parameters (both enthalpy and entropy) of the rate-limiting steps, as monitored by tyrosine absorbance and fluorescence, are identical for the refolding of urea- and Gdn·HCl-denatured RNase A. A refolding scheme involving one intermediate on each of the two slow-folding pathways is proposed by adopting the notion that RNase A refolds through a sequential mechanism. However, these two intermediates are formed from their respective unfolded forms (U_S^{II} and U_S^{I}) through two different processes of distinct physical origin. The intermediate I_N, which is formed from the major slow-folding species U_S^{II} through a conformational folding step, already possesses many properties of the native protein. In contrast, the intermediate (designated as I′) on the minor slow-folding pathway is formed from U_S^{I} by the isomerization of a proline residue (possibly Pro^{93}) and is still conformationally unfolded. It is shown that such a refolding scheme can account for the known kinetic features of *both* major and minor slow-refolding pathways of RNase A. Specifically, it can account for the following experimental observations: (i) the merging of the major and minor slow-folding phases into a single one at high concentrations of denaturant; (ii) the retention of the biphasic behavior of the slow-folding kinetics at high concentrations of denaturant when $(NH_4)_2SO_4$ is added; and (iii) the reversal of the relative amplitudes of the two phases when detected by tyrosine fluorescence as compared to UV absorbance. As an alternative to proline isomerization, it is also hypothesized that the stereochemical inversion of the chirality of a disulfide bond in the protein molecule might be the underlying molecular basis for the $I_N \rightarrow N$ step.

The existence of multiple unfolded forms of bovine pancreatic ribonuclease A (RNase A)[1] in its denatured state is now well established (Garel & Baldwin, 1973; Garel et al., 1976; Hagerman & Baldwin, 1976). From its biphasic refolding kinetics, it has been concluded that the different unfolded forms interconvert slowly and refold to the native enzyme on vastly different time scales, ranging from milliseconds to seconds. These unfolded forms are designated as fast-folding (U_F) and slow-folding (U_S) species and exist at an equilibrium ratio of 20:80 in the denatured state. To account for the slow interconversion between the different unfolded conformations and the difference in refolding rates, Brandts et al. (1975) proposed that, while all of the prolyl residues of U_F are in their native conformations, U_S contains one or more nonnative proline conformations and the cis–trans isomerization(s) of these residues is (are) responsible for the slow-refolding kinetics. Since then, considerable experimental evidence has been accumulated in support of the involvement of proline isomerization in the folding/unfolding of RNase A (Nall et al., 1978; Schmid & Baldwin, 1978, 1979a,b) and of other proteins (Ko et al., 1977; Crisanti & Matthews, 1981; Desmadril & Yon, 1981; McPhie, 1982; Goto & Hamaguchi, 1982; Nall, 1983).

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A with intact disulfide bonds; U_S and U_F, slow- and fast-folding species of denatured RNase A, respectively; BPTI, bovine pancreatic trypsin inhibitor; Gdn·HCl, guanidine hydrochloride; NaOAc, sodium acetate; 2′-CMP, cytidine 2′-phosphate; CD, circular dichroism; NMR, nuclear magnetic resonance; Gly·HCl, glycine hydrochloride.

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