MacDonald, R. J., Stary, S. J., & Swift, G. H. (1982) *J. Biol. Chem. 257*, 9724–9732.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, pp 466–467, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mason, A. J., Evans, B. A., Cox, D. R., Shine, J., & Richards, R. I. (1983) *Nature (London) 303*, 300–307.
Maxam, A., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
Nustad, K., Orstavik, T. B., Gautvik, K. M., & Pierce, J. V. (1978) *Gen. Pharmacol. 9*, 1–9.
Orstavik, T. B., & Glenner, G. G. (1978) *Acta Physiol. Scand. 103*, 384–393.
Orstavik, T. B., Nustad, K., Brandtzaeg, P., & Pierce, J. V. (1976) *J. Histochem. Cytochem. 24*, 1037–1039.
Rigby, P. J. W., Dieckman, M., Rhodes, C., & Berg, P. (1977) *J. Mol. Biol. 113*, 237–251.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Stark, G. R., & Williams, J. G. (1979) *Nucleic Acids Res. 6*, 195–203.
Stellwag, E. J., & Dahlberg, A. E. (1980) *Nucleic Acids Res. 8*, 299–317.
Suggs, S. V., Hirose, T., Miyake, T., Kawashima, E. H., Johnson, M. J., Itakura, K., & Wallace, B. (1981) in *Developmental Biology Using Purified Genes* (Brown, D. D., Ed.) pp 683–693, Academic Press, New York.
Swift, G. H., Dagorn, J.-C., Ashley, P. L., Cummings, S. W., & MacDonald, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7263–7267.
Thomas, K. A., Baglan, N. C., & Bradshaw, R. A. (1981) *J. Biol. Chem. 256*, 9156–9166.
Wallace, R. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T., & Itakura, K. (1979) *Nucleic Acids Res. 6*, 3543–3557.

Quantitative Determination of the 5-(Hydroxymethyl)uracil Moiety in the DNA of γ-Irradiated Cells†

Krystyna Frenkel,‡ Archie Cummings,‡ Jerome Solomon,§ Jean Cadet,‖ Jacob J. Steinberg,‡ and George W. Teebor*,‡

Departments of Pathology and Environmental Medicine, New York University Medical Center, New York, New York 10016, and Laboratoires de Chimie, Departement de Recherche Fondamentale, Centre d'Etudes Nucleaires de Grenoble, 85X, 38041 Grenoble Cedex, France

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ABSTRACT: 5-(Hydroxymethyl)uracil (HMUra) is a chemically stable derivative of thymine formed through the action of ionizing radiation which we previously identified in the DNA of γ-irradiated HeLa cells [Teebor, G. W., Frenkel, K., & Goldstein, M. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 318–321]. In this report, we determine whether HMUra can be used as a marker of exposure of DNA to ionizing radiation. Dose–response curves for its formation in [^{3}H]thymidine-labeled DNA were constructed by exposing the DNA to increasing amounts of γ-radiation and measuring the HMUra content. DNA was irradiated both in solution and in intact cells. HMUra was identified as the 2′-deoxyribonucleoside 5-(hydroxymethyl)-2′-deoxyuridine (HMdU) by subjecting the irradiated DNA to enzymatic digestion and analyzing the mixture of 2′-deoxyribonucleosides by high-pressure liquid chromatography. The identity of the radiogenically formed HMdU was confirmed by acetylation and the structure of the acetyl derivative obtained by mass and nuclear magnetic resonance spectroscopies. At two different DNA concentrations in solution, the same number of thymidine moieties were converted to HMdU, indicating that within this range of concentration the formation of HMdU was mediated through the indirect action of ionizing radiation. Equal amounts of HMdU were formed in single- and double-stranded DNA at each radiation dose, indicating that DNA conformation did not affect HMdU formation. Surprisingly, the *G* value (number of HMdU molecules formed/100 eV) was higher in irradiated cellular DNA than in DNA irradiated in solution. This may be due to the fact that although the thymine moiety in DNA in solution is more vulnerable to hydroxyl radical attack than in the cell, the radiogenically formed 5-methyleneuracil radical re-forms thymine through reaction with hydrogen radicals more readily in solution than in the cell, resulting in a small net yield of HMdU. HMdU was formed in a dose-dependent manner whether the DNA was irradiated in solution or in intact cells. We conclude that HMdU may serve as a quantitative marker of exposure of the genetic material of living cells to ionizing radiation.

The carcinogenic and mutagenic properties of ionizing radiation are thought to be a direct consequence of the damage it causes to DNA (Upton, 1975). Therefore, it is important to find a quantitative marker of radiation damage which could serve in a manner analogous to the cyclobutane pyrimidine dimer induced by UV radiation (Carrier & Setlow, 1971). The damage to DNA effected by ionizing radiation is heterogeneous and includes single- and double-strand breaks, DNA–protein cross-links, loss of bases from the DNA backbone, and chemical modification of the bases remaining on the DNA backbone (Rhaese & Freese, 1968; Dunlap & Cerutti, 1975;

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‡ Department of Pathology.
§ Department of Environmental Medicine.
‖ Departement de Recherche Fondamentale.

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Ward & Kuo, 1976; Cadet et al., 1981a,b; Mee & Adelstein, 1981; Olinski et al., 1981; Henner et al., 1982; Cress & Bowden, 1983). Of these differing types of damage, modified bases seem to be the most suitable as markers of radiation exposure since they are chemically distinct from normal constituents of DNA and, therefore, are readily identifiable. Indeed, many types of modified bases have been identified as products of the irradiation of solutions of bases, nucleosides, and nucleotides, and DNA itself [Scholes et al., 1960; reviewed by Scholes (1976)]. However, until recently, it was not possible to identify these modified bases by direct chemical analysis of the DNA of irradiated cells. The sensitivity and resolving capacity of high-pressure liquid chromatography (HPLC)[1] coupled with the synthesis of marker compounds enabled us to identify several radiogenic thymine derivatives in cellular DNA (Frenkel et al., 1981a,b; Teebor et al., 1982, 1984). Among these was HMUra which is thought to be formed through the attack of radiogenically derived hydroxyl radicals on the methyl group of thymine (Roti Roti & Cerutti, 1974; Roti Roti et al., 1974; Swinehart & Cerutti, 1975; Cerutti, 1976). HMUra had first been identified as a minor radiation product of thymine irradiated in solution (Ekert, 1962). Subsequently, it was shown that its formation was favored with increasing pH (Myers et al., 1965). Its radiogenic formation in cellular DNA was inferred through the demonstration of the generation of ^3H_2O after irradiation of CHO cells whose DNA had been prelabeled with [*methyl*-^{3}H]dT (Roti Roti & Cerutti, 1974).

In this report, we describe methodology with which HMUra can unambiguously be identified and quantitatively measured in DNA irradiated in solution and in intact cells. Using this methodology, we prove the suitability of HMUra as a marker of exposure of DNA to ionizing radiation.

Experimental Procedures

Materials

[6-^{3}H]dT (19.3 Ci/mmol) was purchased from New England Nuclear. HMdU, DNase I, spleen phosphodiesterase, snake venom phosphodiesterase, alkaline phosphatase, and S_1 nuclease from *Aspergillus oryzae* were purchased from Sigma. *Escherichia coli* W3110 (*thy*$^-$), J. Cairns strain, was obtained from the *E. coli* Genetic Stock Center at Yale University School of Medicine, New Haven, CT. Growth medium was purchased from GIBCO. HPLC-grade acetone and acetonitrile were purchased from Fisher.

Methods

Preparation of E. coli [^{3}H]DNA. DNA was radioactively labeled and then isolated from *E. coli* W3110 (*thy*$^-$), J. Cairns strain, as previously described (Frenkel et al., 1981a,b), except [6-^{3}H]dT was used instead of [*methyl*-^{3}H]dT to avoid formation of [^{3}H]HMdU through transmutation (Teebor et al., 1984). The specific activity of the DNA was 1.6×10^4 dpm/μg assuming that $1A_{260}$ unit = 50 μg of DNA/mL.

γ-Irradiation of [^{3}H]DNA. DNA was drop dialyzed (Millipore VS 0.025 μm) for 1 h into water and half of the DNA denatured by heating at 100 °C for 10 min followed by rapid cooling in an ice–water bath. After appropriate dilutions, solutions of single-stranded (ss) and double-stranded (ds) DNA at 25 and 250 μg/mL kept at room temperature were irradiated under air from a ^{137}Cs source at a dose rate of 450–475 rd/min as measured by thermoluminescent dosimeters (Harshaw), calibrated against a standard ^{60}Co source.

S_1 Digestion. Solutions containing ss and ds nonirradiated and irradiated DNAs were treated with S_1 nuclease (33.3 units/100 μL) after addition of buffer, yielding a final concentration of 20 mM NaOAc, 50 mM NaCl, and 1 mM $ZnSO_4$, pH 4.5. Samples were incubated with S_1 at 37 °C for 20 min, and after addition of 100 μL of BSA (1 mg/mL H_2O), enzyme and undigested DNA were precipitated with 200 μL of cold 10% Cl_3CCOOH. After 15 min at 4 °C, the samples were centrifuged (Sorvall RC2 with SS-34 rotor) at 7000 rpm for 10 min, and half the volume of the supernatant was counted in HP Redi-Solv (Beckman) in a Nuclear Chicago Mark II scintillation counter.

γ-Irradiation of HeLa Cells and Isolation of DNA. HeLa cells grown in suspension culture were labeled with [6-^{3}H]dT, washed with cold phospate-buffered saline, and γ-irradiated from the ^{137}Cs source. After irradiation, cells were harvested, and the DNA was extracted (Teebor et al., 1984). The specific activity of the DNA was between 1.2×10^5 and 1.5×10^5 dpm/μg. The nonirradiated cells were processed in the same manner and used as controls. Approximately 10^8 cells were used for each radiation dose point. This number of cells yielded about 0.8 mg of [^{3}H]DNA.

Enzymatic Hydrolysis of DNA. Buffer was added to the control and irradiated DNA samples obtained from either *E. coli* or HeLa cells to a final concentration of 0.1 M NaCl and 0.01 M Tris-HCl, pH 8.0, and the DNA was digested to 2′-deoxyribonucleosides (Frenkel et al., 1981a,b). The hydrolysates were precipitated with acetone, and after centrifugation (Sorvall RC-2 with SS-34 rotor) at 7000 rpm for 10 min, the supernatants were evaporated to dryness. The residues were dissolved in water, filtered through a Millipore 0.22-μm pore diameter filter, and, after mixing with appropriate markers, analyzed by HPLC (Frenkel et al., 1981b).

HPLC Analysis. Enzymatic hydrolysates of DNA labeled with [6-^{3}H]dT [$(1–2) \times 10^6$ cpm for DNA irradiated in solution; $(2–4) \times 10^7$ cpm for cellular DNA] were chromatographed together with marker compounds including authentic HMdU on a 5-μm Ultrasphere-ODS column (Altex, 1 × 25 cm) with water as eluent using a Beckman HPLC Model 330 equipped with a gradient elution accessory and a microprocessor–controller (Model 421). The flow rate was 2 mL/min. Fractions were collected at 1-min intervals. To elute dT, an acetonitrile–water gradient was used starting with fraction 61.

Acetylation and HPLC Analysis of Acetyl Derivatives. Fractions containing ^{3}H from the DNA digest which cochromatographed with authentic marker HMdU were combined, evaporated to dryness, dissolved in dry pyridine, and acetylated with acetic anhydride. After 15 h (overnight) at room temperature, the unreacted acetic anhydride was decomposed by addition of water. Samples were evaporated to dryness under reduced pressure, and the residue was dissolved in 25% acetonitrile–H_2O, filtered through a Millipore filter, and chromatographed again on the ODS column with 25% acetonitrile–water as the eluent at a flow rate of 2 mL/min. The same acetylation and purification procedures were used for the preparation of the acetyl derivative of authentic HMdU in milligram amounts needed for the MS and NMR analyses. Before acetylation, pyridine (HPLC grade, Mallinckrodt) was refluxed over calcium hydride for 4 h and then distilled into an amber-colored flask containing molecular sieves. Acetic anhydride (Fisher) was distilled prior to use.

[1] Abbreviations: HMUra, 5-(hydroxymethyl)uracil; HMdU, 5-(hydroxymethyl)-2′-deoxyuridine; T, thymine; dT, thymidine; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; NMR, nuclear magnetic resonance; MS, mass spectroscopy; Cl_3CCOOH, trichloroacetic acid; EI, electron impact; CI, chemical ionization; CHO, Chinese hamster ovary.

Table I: ^{1}H Chemical Shifts[a] (ppm) of 5-(Hydroxymethyl)-2′-deoxyuridine and Its 3′,5,5′-Tri-O-acetylated Derivative in CD_3OD[b]

nucleoside	position									$COCH_3$	
	1′	2′	2″	3′	4′	5′	5″	5	6	5	3′,5′
HMdU	6.28	2.22	2.28	4.39	3.92	3.78	3.72	4.31	7.94		
3′,5′,5-tri-*O*-acetoxy-HMdU	6.22	2.34	2.48	5.26	4.28	4.30	4.37	4.82	7.83	2.00	2.09, 2.11

[a] Spectra obtained on a CAMECA TSN spectrometer at 250 MHz (20 °C). The iterative program LAOCOON was used for the spectral analysis. [b] The chemical shifts have been measured in CD_3OD from the internal reference tetramethylsilane-d_4 and are estimated to be accurate to within 0.01 ppm.

Purification of [^{3}H]HMdU Formed from [methyl-^{3}H]dT through Transmutation (Teebor et al., 1984). Samples of [*methyl*-^{3}H]dT which were several months old were chromatographed on the ODS column with authentic HMdU as the UV marker and water as eluent. ^{3}H-Containing fractions which coeluted with the marker were concentrated, rechromatographed, and concentrated again. Known amounts of this [^{3}H]HMdU were acetylated to determine the yield of acetylation.

Mass Spectra. Electron-impact (EI) and isobutane chemical ionization (CI) mass spectra were acquired on a Du Pont 21-492 high-resolution mass spectrometer equipped with a dual EI/CI ionization source. The source temperature was maintained at 250–265 °C. Samples were introduced as solids. EI spectra were obtained with introduction via the direct probe with the probe temperature kept at the minimum needed to volatilize the sample. Isobutane CI spectra were obtained by using desorption from an extended Vespel probe (Cotter, 1980). Matheson instrument grade isobutane (99.5%) was used and kept at 0.5-torr ion-source pressure.

The CI spectrum of HMdU contains an intense protonated molecular ion (MH^+) at m/z 259, establishing the molecular weight of 258. This ion fragments through the sequential loss of two water molecules, resulting in peaks at m/z 241 ($MH^+ - H_2O$) and 223 ($MH^+ - 2H_2O$). The most intense peak in the CI spectrum (m/z 143) results from cleavage of the glycosyl bond and subsequent hydrogen transfer which gives an ion containing the base plus two hydrogens (bH_2). This ion further decomposes, losing water, to form m/z 125 ($bH_2 - H_2O$). The typical ions of the deoxyribose moiety in the CI spectra (McCloskey, 1974) are found at m/z 117 (S), 99 (S $- H_2O$), and m/z 81 (S $- 2H_2O$) where S represents the sugar. The spectrum of acetylated HMdU indicates that derivatization occurred at the hydroxyl group of the base and two hydroxyls of 2-deoxyribose, resulting in a triacetate whose molecular weight is 384. The spectrum has only a small MH^+ at m/z 385 due to the facile loss of acetic acid (AcOH) moieties. Loss of one AcOH gives a signal at m/z 325 (MH^+ – AcOH) and of three AcOH at m/z 205 (MH^+ – 3AcOH). The ions associated with the base are small with a minor peak at m/z at 185 (bH_2) which loses AcOH to form m/z 125 (bH_2 – AcOH). The most abundant ions are due to the acetylated sugar at m/z 201 which sequentially loses one and two molecules of AcOH to form m/z 141 (S – AcOH) and 81 (S – 2AcOH), respectively.

NMR Spectra. Both authentic HMdU and its acetylated derivative were HPLC purified prior to NMR analysis. The 250-MHz ^{1}H Fourier-transform NMR spectra were obtained on a CAMECA TSN 250 spectrometer. The samples were evaporated twice from 99.5% methanol-d_4 and dissolved in 99.9% methanol-d_4 (Commissariat a l'Energie Atomique, Saclay, France) to a concentration of 1% (w/w). The ^{1}H chemical shift data were measured relative to internal tetramethylsilane at a probe temperature of 20 ± 1 °C. Iterative spectroscopic analyses were made by utilizing the LAOCOON III program, and the final computer-simulated spectra were obtained with the CACTUS 5 program.

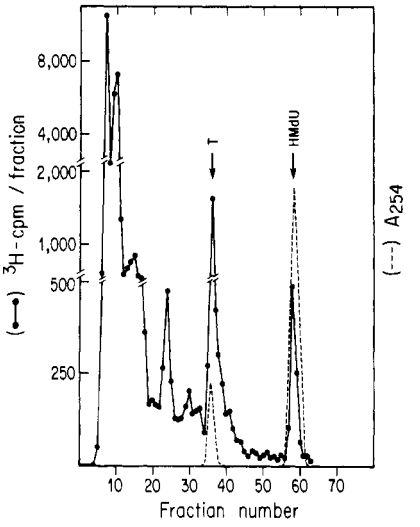

FIGURE 1: HPLC analysis of an enzymatic hydrolysate (2′-deoxyribonucleosides) of γ-irradiated (42 krd) [6-^{3}H]dT-labeled ssDNA (●) in the presence of authentic T and HMdU (---).

The ^{1}H NMR spectra were analyzed by using computer-simulated spectra which facilitated calculations of various parameters including chemical shifts and coupling constant values. The 250-MHz ^{1}H NMR spectrum of HMdU in methanol-d_4 exhibits many of the features reported in the literature for this compound (Baker et al., 1966; Birnbaum et al., 1980; Shiay et al., 1980). Table I lists chemical shifts of HMdU and its acetyl derivative. The spectrum of the derivative showed three additional singlets in the range of 2.00–2.11 ppm which are characteristic of an acetylated nucleoside. The two upfield signals (δ 2.09 and 2.11) were assigned to the resonance signals of the two acetoxy groups at osidic carbons C(3′) and C(5′) by analogy with 3′,5′-di-*O*-acetoxythymidine (δ 2.08 and 2.12). In addition, the electronegative acetoxy groups had a pronounced deshielding effect on the methinic protons, as observed for H(3′) (Δδ 0.51), H(5′) (Δδ 0.52), and H(5″) (Δδ 0.65), and a somewhat smaller effect for H(4′) (Δδ 0.36). The remaining (δ 2.00) signal was assigned to the acetoxy group on the methylene group of C(5).

RESULTS

HPLC Analysis of DNA Hydrolysates. A representative HPLC profile of irradiated *E. coli* DNA is depicted in Figure 1. The hydrolysate derived from about 250 μg of DNA (2.0×10^6 cpm) which had been exposed to 42 krd of γ-radiation was applied to the ODS column. The HPLC profile of nonirradiated DNA (not shown) was at the base line until all the radioactivity appeared as dT. Irradiation of DNA resulted in the appearance of several early eluting radiation products of T which have not yet been unambiguously identified but whose retention times we know are those of thymine glycol and its oxidation products *N*′-formyl-*N*-pyruvylurea and 5-hydroxy-5-methylhydantoin and the free base HMUra (Teebor

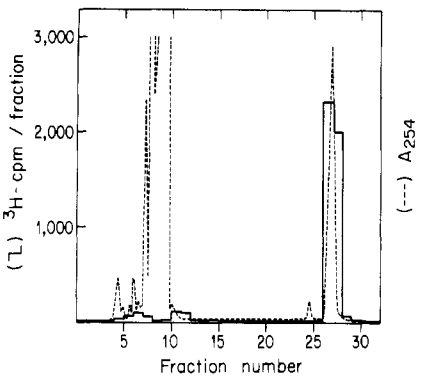

FIGURE 2: Chromatography of acetylated pooled HPLC fractions 57–61 (Figure 1) containing authentic marker HMdU (---) and radiogenically derived [^{3}H]HMdU (—).

et al., 1982). A ^{3}H-containing peak eluted with authentic marker T (fractions 36–38) and another with authentic marker HMdU (fractions 57–61). The remaining radioactivity (about 90%) was recovered as dT which was eluted with an acetonitrile–water gradient begun after fraction 61 (HMdU) and is not included in the graph.

Acetylation of HMdU. To prove that the ^{3}H-containing peak coincident with the UV absorption of authentic HMdU was indeed radiogenically formed HMdU, fractions 57–61 were pooled and acetylated, and the mixture was again analyzed by HPLC. Figure 2 depicts the profile of these acetylated fractions showing a single ^{3}H-containing peak coincident with a single UV-absorbing peak (fractions 27–28). The early eluting UV-absorbing material contains pyridine salts. To confirm that the UV-absorbing peak was the acetyl derivative of HMdU, authentic HMdU was acetylated and analyzed by HPLC. A single UV-absorbing peak was identified at the same retention time as that shown in Figure 2.

Structure of the Acetyl Derivative of HMdU. The structure of this acetyl derivative was determined by mass and NMR spectroscopies. The spectral characteristics of HMdU were obainted for comparison. The MS EI spectra gave no molecular weight information because the molecular ion fragmented to low mass peaks. The CI spectra of HMdU and acetylated HMdU offered complete structural identification (Figure 3) which was confirmed by NMR (Table I), proving the derivative to be 3′,5′,5-tri-*O*-acetoxy-HMdU.

Yield of Acetylation. The yield of acetylation was determined by acetylating known amounts of [^{3}H]HMdU which was obtained from solutions of aging [*methyl*-^{3}H]dT as the transmutation product. Such [^{3}H]HMdU was subjected to chromatographic analysis, as in Figure 1, followed by acetylation and analysis, as in Figure 2. The final recovery was between 70% and 80%.

Dose–Response Curve for Formation of HMdU in DNA Irradiated in Solution and Calculation of G Value. Dose–response curves for the formation of HMdU in irradiated solutions of [6-^{3}H]dT-labeled DNA were constructed. The percent HMdU formed was expressed as the amount of [^{3}H]HMdU recovered as the acetylated derivative (Figure 2) divided by 0.75 (the average yield of acetylation), then divided by the total ^{3}H of the DNA hydrolysate injected into the ODS column under the conditions of Figure 1, and multiplied by 100.

To determine whether the formation of HMdU was primarily mediated through the indirect action of ionizing radiation, two concentrations of DNA (25 and 250 μg/mL) were irradiated. The HMdU content of the 250 μg/mL samples, measured as percent, was an order of magnitude lower than that irradiated at 25 μg/mL. This means that at both concentrations the same absolute number of HMdU molecules were formed per unit volume of solution, proving that the formation of HMdU was the result of the indirect action of the ionizing radiation. In irradiated solutions, a fixed number of free radicals are produced by a given dose. Therefore, the number of solute molecules altered (in this case, dT converted to HMdU) is independent of their concentration so that the percent modification decreases with increasing concentration of DNA (Bacq & Alexander, 1961). On the basis of the data shown in Figure 4, the *G* value for the formation of HMdU in DNA irradiated in solution was calculated. The *G* value (number of HMdU molecules formed/100 eV) is 0.002.

Effect of DNA Conformation on HMdU Formation. Both ss- and dsDNA were irradiated at each dose indicated on the abscissa in Figure 4 to determine whether DNA conformation affected the formation of HMdU. The standard errors of the

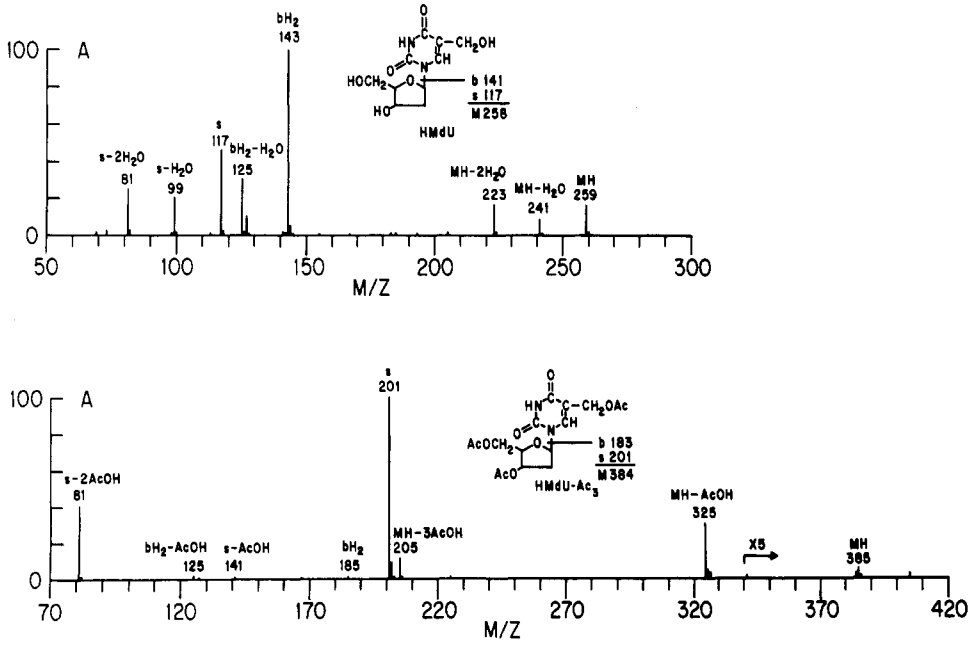

FIGURE 3: Mass spectra of HMdU and triacetoxy-HMdU.

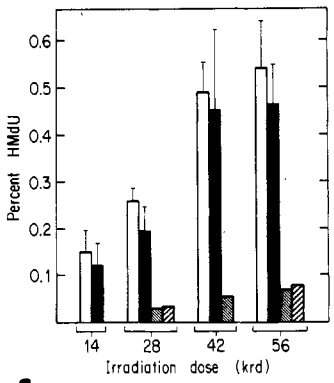

FIGURE 4: Percent HMdU formed in γ-irradiated [^{3}H]DNA. At 25 μg of DNA/mL: (□) ssDNA and (■) dsDNA. At 250 μg of DNA/mL: (▧) ssDNA and (▨) dsDNA. Vertical lines represent standard error of the means.

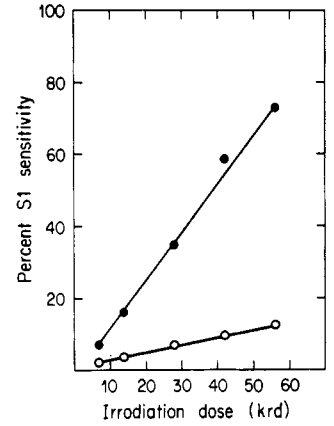

FIGURE 5: Sensitivity of [^{3}H]dsDNA γ-irradiated at 25 μg of DNA/mL (●) and at 250 μg of DNA/mL (○) to digestion by S_1 nuclease.

mean were calculated for three to seven determinations per point. It can be seen that the HMdU content increased in a relatively linear fashion, leveling off at the highest radiation doses. Student's *t* test applied to each set of radiation dose matched points for ss- and dsDNA indicated that there were no significant differences between the two means. This suggested that the radiogenic formation of HMdU was not affected by the conformation of the DNA.

However, since it was possible that the irradiation of dsDNA rendered it single stranded (Teoule et al., 1977), samples of dsDNA (both concentrations) were irradiated at the same radiation doses as in Figure 4, and then the DNA was subjected to S_1 nuclease digestion. This enzyme hydrolyzes ssDNA and renders it acid soluble. Figure 5 shows that the DNA did indeed become progressively more S_1 sensitive as the radiation dose increased. However, at 14 and 28 krd, the DNA irradiated at 25 μg/mL was still primarily double stranded. Since the formation of HMdU was the same in ss- and dsDNA at these doses, it may definitely be concluded that conformation did not affect HMdU formation. It should also be noted that, like the formation of HMdU, the S_1 sensitivity of the DNA irradiated at 250 μg/mL was also significantly lower than that at 25 μg/mL, indicating that about the same number of enzyme-sensitive sites were formed at the two DNA concentrations.

Dose–Response Curves for Formation of HMdU in Cellular DNA and Calculation of G Value. HeLa cells which had been prelabeled with [6-^{3}H]thymidine were exposed to increasing amounts of radiation (14, 28, and 56 krd). Their DNA was then extracted, subjected to enzymatic digestion, and analyzed by HPLC in a manner identical with that shown for irradiated *E. coli* DNA in Figure 1. The fractions eluting with authentic HMdU were pooled, acetylated, and analyzed as shown in Figure 2. A recovery of 75% was assumed as for *E. coli* DNA. These experiments indicated that for 14, 28, and 56 krd of γ-radiation, the percent HMdU formed in HeLa cell DNA was 0.0006, 0.0013, and 0.0039, respectively. Taking the average DNA content of a HeLa cell nucleus to be 16×10^{-12} g, and the average diameter of the HeLa nucleus to be 8 μm with a density of 1, the *G* value obtained for the formation of HMdU in cellular DNA is 0.03.

Discussion

These experiments show that HMUra is formed in DNA exposed to ionizing radiation, be it in solution or in the cell, in a dose-dependent manner which is relatively linear. Thus, the HMUra moiety, measured as HMdU in enzymatic hydrolysates of DNA, serves as a marker of exposure of DNA to ionizing radiation just as the cyclobutane pyrimidine dimer, isolated from acid hydrolysates of DNA, serves as a marker of exposure of DNA to UV radiation (Carrier & Setlow, 1971). Like the pyrimidine dimer, the quantitative identification of HMdU is currently limited to those experimental conditions in which the DNA, be it irradiated in solution or in the cell, is prelabeled with [^{3}H]dT. [6-^{3}H]dT rather than [*methyl*-^{3}H]dT must be used since HMdU is also formed as the transmutation product of the latter radionuclide (Teebor et al., 1984), thus precluding accurate measurement of its formation through the action of ionizing radiation. The use of microderivatization is necessary for cellular studies since high levels of [^{3}H]dT derived from the hydrolysis of DNA must be applied to the HPLC column to isolate and identify the relatively small amount of [^{3}H]HMdU formed. Under such conditions, small extraneous ^{3}H-containing peaks occasionally appear near the UV marker HMdU which make it difficult to measure the HMdU content of the irradiated DNA from analysis of the first chromatogram alone. It is possible to store the acetyl derivative of HMdU for several days prior to analysis as long as it is dry. Thus, it is feasible to perform several initial chromatographic analyses as shown in Figure 1, isolate the HMdU fractions and derivatize, and then quantitatively measure the ^{3}H content of the acetates several days later under the conditions shown in Figure 2. When smaller amounts of [^{3}H]dT are present in the hydrolysate such as in the analysis of DNA irradiated in solution, extraneous ^{3}H-containing peaks are usually absent, and the HMdU content of the DNA can be determined from the first chromatogram alone.

In addition to demonstrating that the formation of HMdU is relatively linear with incident radiation dose, the data of Figure 4 also indicate that the formation of HMdU results from the indirect action of ionizing radiation. This is corroborative of the proposed mechanism of its formation through hydroxyl radical attack on the methyl group of T, resulting in formation of the 5-methyleneuracil radical (Roti Roti & Cerutti, 1974; Roti Roti et al., 1974; Swinehart & Cerutti, 1975; Cerutti, 1976) which then reacts with additional hydroxyl radicals to form the chemically stable HMdU. In light of this mechanism, it is not surprising that DNA conformation does not influence the radiogenic formation of HMdU since the methyl group of T lies in the major groove of the DNA helix and is therefore equally susceptible to hydroxyl radical attack be it in ss- or dsDNA.

The data obtained also allowed the calculation of *G* values for the formation of HMdU in irradiated cellular DNA and in DNA irradiated in solution. The *G* value of 0.03 for its

formation in HeLa cells is in very good agreement with that of 0.015 for its formation in CHO cells which was estimated from the measurement of ^3H_2O formed after irradiation of CHO cells whose DNA had been prelabeled with [*methyl*-^{3}H]dT (Roti Roti & Cerutti, 1974). The latter *G* value was calculated by assuming that the formation of ^3H_2O was stoichiometrically related to the liberation of tritium atoms from the methyl group of T as the result of attack by radiogenically derived hydroxyl radicals and the subsequent exchange of the tritium with the hydrogen of water.

Our finding that the *G* value for HMdU formation in HeLa cells (0.03) was higher than in DNA irradiated in solution (0.002) is surprising since it has been widely held that the intracellular milieu is replete with free radical scavengers which protect the DNA from free radical attack (Cerutti, 1976). Indeed, in experiments measuring the amount of ^3H_2O generated from irradiation of [*methyl*-^{3}H]dT-labeled DNA in solution, it was estimated that DNA in solution was almost 4 orders of magnitude more susceptible to hydroxyl radical attack at the methyl group of T than the DNA of the cell (Cerutti, 1976). It is possible that the difference in *G* values is due in part to a chemical restitution reaction (Roti Roti et al., 1974; Cerutti, 1976). It may be that although hydroxyl radical attack on DNA which has been irradiated in solution at relatively dilute concentrations yields many 5-methyleneuracil radicals, most of these re-form the thymine moiety through a reverse reaction with hydrogen atoms resulting in a small net formation of HMdU. In the highly concentrated environment of the nucleus, this restitution reaction might not be as efficient, and therefore, the formation of the HMUra moiety is favored. The hypothesis that the radiogenic formation of HMdU is favored at higher DNA concentrations is further supported by the report of a *G* value of 0.05 which was obtained by immunological measurement of the HMUra moiety in irradiated DNA (Lewis et al., 1978). That DNA had been irradiated at the relatively high concentration of 2.5 mg/mL compared to the 25 and 250 μg/mL used in our experiments which yielded the lower *G* value of 0.002. The independence of the *G* value from DNA concentration which we observed at the more dilute concentrations, a characteristic of the indirect action of radiation, may therefore be attributed to the constancy of the restitution reaction when water was in great excess.

The contribution of radiogenically formed HMUra to the deleterious biological effects of ionizing radiation is as yet uncertain, but it seems significant that HMdU administered as the nucleoside to cells in culture or to animals is incorporated into DNA where it exerts cytostatic and cytotoxic effects (Meldrum et al., 1974; Waschke et al., 1975). It has also been shown to be mutagenic in bacterial systems (Cadet et al., 1981b). Although a normal DNA constituent of some *Bacillus subtilis* phages (Kallen et al., 1962; Okubo et al., 1964; Roscoe & Tucker, 1964), its harmful potential to eukaryotic DNA is further corroborated by the recent demonstration of a repair *N*-glycosylase activity in mouse cells directed against the HMUra moiety in DNA (Hollstein et al., 1984). The existence of a repair enzyme also suggests that HMUra, like radiogenic thymine derivatives such as thymine glycol (Cathcart et al., 1984), may also be formed through endogenous oxidative mechanisms which have been implicated in the etiology of human cancer.

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REFERENCES

Bacq, Z. M., & Alexander, P. (1961) in *Fundamentals of Radiobiology*, pp 45–55, Pergamon Press, Oxford, London, New York, and Paris.

Baker, B. R., Schwan, T. J., & Santi, D. V. (1966) *J. Med. Chem. 9*, 66–72.

Birnbaum, G. I., Deslauriers, R., Lin, T.-S., & Prusoff, W. H. (1980) *J. Am. Chem. Soc. 102*, 4236–4241.

Cadet, J., Berger, M., & Teoule, R. (1981a) *Sov. J. Quantum Electron. (Engl. Transl.) 11*, 1576–1582.

Cadet, J., Voituriez, L., Seed, J. L., Bueding, E., Nagamatsu, T., & Wang, S. Y. (1981b) in International Round Table on Nucleosides, Nucleotides and Their Biological Applications, 4th, Antwerp, Belgium.

Carrier, W. L., & Setlow, R. B. (1971) *Methods Enzymol. 21*, 230–237.

Cathcart, R., Schwiers, E., Saul, R. L., & Ames, B. N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5633–5637.

Cerutti, P. A. (1976) *Photochem. Photobiol. Nucleic Acids 2*, 375–401.

Cline, R. E., Fink, R. M., & Fink, K. (1959) *J. Am. Chem. Soc. 81*, 2521–2527.

Cotter, R. J. (1980) *Anal. Chem. 52*, 1589 (Abstract).

Cress, A. E., & Bowden, G. T. (1983) *Radiat. Res. 95*, 610–619.

Dunlap, B., & Cerutti, P. (1975) *FEBS Lett. 51*, 188–190.

Ekert, B. (1962) *Nature (London) 194*, 278–279.

Frenkel, K., Goldstein, M. S., Duker, N., & Teebor, G. W. (1981a) *Biochemistry 20*, 750–754.

Frenkel, K., Goldstein, M. S., & Teebor, G. W. (1981b) *Biochemistry 20*, 7566–7571.

Henner, W. D., Grunberg, S. M., & Haseltine, W. A. (1982) *J. Biol. Chem. 257*, 11750–11754.

Hollstein, M. C., Brooks, P., Linn, S., & Ames, B. N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4003–4007.

Kallen, R. G., Simon, M., & Marmm, J. (1962) *J. Mol. Biol. 5*, 248–250.

Lewis, H. L., Muhleman, D. R., & Ward, J. F. (1978) *Radiat. Res. 75*, 305–316.

McCloskey, J. M. (1974) *Basic Princ. Nucleic Acid Chem. 17*, 209–309.

Mee, L. K., & Adelstein, S. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2194–2198.

Meldrum, J. B., Gupta, V. S., & Saunders, J. R. (1974) *Antimicrob. Agents Chemother. 6*, 393–396.

Myers, L. S., Jr., Ward, J. F., Tsukamoto, W. T., Holmes, D. E., & Julca, J. R. (1965) *Science (Washington, D.C.) 148*, 1234–1235.

Okubo, S., Strauss, B., & Stodolsky, M. (1964) *Virology 24*, 552–562.

Olinski, R., Briggs, R. C., Hurilica, L. S., Stein, T., & Stein, G. (1981) *Radiat. Res. 86*, 102–114.

Rhaese, H.-J., & Freese, E. (1968) *Biochim. Biophys. Acta 155*, 476–490.

Roscoe, D. H., & Tucker, R. G.(1964) *Biochem. Biophys. Res. Commun. 16*, 106–110.

Roti Roti, J. L., & Cerutti, P. A. (1974) *Int. J. Radiat. Biol. 25*, 413–417.
Roti Roti, J. L., Stein, G. S., & Cerutti, P. A. (1974) *Biochemistry 13*, 1900–1905.
Scholes, G. (1976) *Photochem. Photobiol. Nucleic Acids 1*, 521–577.
Scholes, G., Ward, J. F., & Weiss, J. (1960) *J. Mol. Biol. 2*, 379–391.
Shiay, G. T., Schinazi, R. F., Chen, M. S., & Prusoff, W. H. (1980) *J. Med. Chem. 23*, 127–133.
Swinehart, J. L., & Cerutti, P. A. (1975) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 27*, 83–94.
Teebor, G. W., Frenkel, K., & Goldstein, M. S. (1982) *Adv. Enzyme Regul. 20*, 39–54.
Teebor, G. W., Frenkel, K., & Goldstein, M. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 318–321.
Teoule, R., Bert, C., & Bonicel, A. (1977) *Radiat. Res. 72*, 190–200.
Upton, A. (1975) in *Cancer* (Becker, F. F., Ed.) pp 387–403, Plenum Press, New York and London.
Ward, J. F., & Kuo, I. (1976) *Radiat. Res. 66*, 485–498.
Waschke, S., Reefschlager, J., Barwolff, D., & Langen, P. (1975) *Nature (London) 255*, 629–630.

Contributions of Dangling End Stacking and Terminal Base-Pair Formation to the Stabilities of XGGCCp, XCCGGp, XGGCCYp, and XCCGGYp Helixes†

Susan M. Freier,‡ Dirk Alkema,§ Alison Sinclair,§ Thomas Neilson,§ and Douglas H. Turner*,‡
Department of Chemistry, University of Rochester, Rochester, New York 14627, and Department of Biochemistry, McMaster University, Hamilton, Ontario L8S 4M1, Canada
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ABSTRACT: The role of stacking in terminal base-pair formation was studied by comparison of the stability increments for dangling ends to those for fully formed base pairs. Thermodynamic parameters were measured spectrophotometrically for helix formation of the hexanucleotides AGGCCUp, UGGCCAp, CGGCCGp, GCCGGCp, and UCCGGAp and for the corresponding pentanucleotides containing a 5′-dangling end on the GGCCp or CCGGp core helix. In 1 M NaCl at 1×10^{-4} M strands, a 5′-dangling nucleotide in this series increases the duplex melting temperature (T_m) only 0–4 °C, about the same as adding a 5′-phosphate. In contrast, a 3′-dangling nucleotide increases the T_m at 1×10^{-4} M strands 7–23 °C, depending on the sequence [Freier, S. M., Burger, B. J., Alkema, D., Neilson, T., & Turner, D. H. (1983) *Biochemistry 22*, 6198–6206]. These results are consistent with stacking patterns observed in A-form RNA. The stability increments from terminal A·U, C·G, or U·A base pairs on GGCC or a terminal U·A pair on CCGG are nearly equal to the sums of the stability increments from the corresponding dangling ends. This suggests stacking plays a large role in nucleic acid stability. The stability increment from the terminal base pairs in GCCGGCp, however, is about 5 times the sum of the corresponding dangling ends, suggesting hydrogen bonding can also make important contributions.

Pairing of complementary bases and stacking of base pairs contribute to nucleic acid stability (Cantor & Schimmel, 1980; Bloomfield et al., 1974). Oligonucleotide helixes with terminal unpaired residues (dangling ends) provide useful model systems to study the role of stacking in nucleic acid stability (Martin et al., 1971; Romaniuk et al., 1978; Neilson et al., 1980; Alkema et al., 1981a,b; Petersheim & Turner, 1983; Freier et al., 1983a, 1984). "Pairing" effects can then be estimated by subtracting the free energy due to stacking from the total free energy of a base pair.

Previously we examined the sequence dependence of the stability increment provided by adding an unpaired nucleotide to the 3′ terminus of GGCC[1] and CCGG and found the melting temperature at 1×10^{-4} M strands increases 7–23 °C in 1 M NaCl (Freier et al., 1983a, 1984). We report below the effects of adding a dangling nucleotide to the 5′ end of GGCC or CCGG and of adding base pairs to both ends of either core. Comparison of these results provides an empirical measure of the contributions of stacking and pairing to the free energy of terminal base pairs.

Materials and Methods

Oligonucleotide Synthesis. AGGC, CGGC, UGG, GCCG, and UCCG were synthesized chemically by using phosphotriester procedures and were characterized by ^{1}H NMR (England & Neilson, 1976; Werstiuk & Neilson, 1976; Alkema et al., 1981a, 1982; Sinclair et al., 1984).

UGGCp was prepared by addition of pCp to UGG using T4 RNA ligase (Uhlenbeck & Cameron, 1977; England & Uhlenbeck, 1978). Following purification of the product, the 3′-phosphate was removed by incubation with calf alkaline phosphatase to yield UGGC. Conditions for the ligase reaction are given by Freier et al. (1983a); conditions for the phosphatase reaction are given below. The pentanucleotides XGGCCp and XCCGGp were synthesized from the respective tetranucleoside triphosphates by using the appropriate 5′–

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‡ University of Rochester.
§ McMaster University.

[1] For oligonucleotides, internal phosphates are not denoted; GGCC is GpGpCpC. If a molecule contains a terminal phosphate, it is explicitly indicated.

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