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Production of Dihydrothymidine Stereoisomers in DNA by γ-Irradiation†

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ABSTRACT: 5,6-Dihydrothymidine (dDHT) is a derivative of thymidine formed during γ-irradiation. This paper demonstrates the conditions under which dDHT is formed in solutions of DNA and that dDHT is produced in the DNA of HeLa cells during γ-irradiation. The product of dDHT by γ-irradiation of either thymidine or DNA has been quantitated by a sensitive and specific high-pressure liquid chromatography method. dDHT is a major product of the anoxic irradiation of thymidine (*G* value 0.5) but is produced in substantially smaller amounts in DNA irradiated under the same conditions (*G* value 0.026). The presence of oxygen reduces the yield of dDHT by at least 25-fold for both irradiation substrates. In HeLa cells, ^{60}Co irradiation under anoxia produces $(6.2 \pm 0.2) \times 10^{-8}$ mol of the *R* isomer of dDHT per mole of cell deoxynucleotide per gray (*G* value 0.11). γ-Irradiation of thymidine produces equal quantities of the *R* and *S* stereoisomers of dDHT. Irradiation of DNA produces significantly more (69%) (*R*)- than (*S*)-dDHT. DNA isolated from cultured human cells following γ-irradiation also contains more of the *R* than the *S* form of dDHT. The conformation of double-stranded DNA favors a stereospecific production of the *R* isomer. Among products of γ-irradiation of DNA, dDHT is unique in its strict requirement for anoxia during irradiation and the preferential production of a particular stereoisomer.

γ-Irradiation of DNA produces a variety of alterations in both the phosphodiester backbone and the component bases (Ward, 1981). Several of the altered bases produced by γ-irradiation in DNA, such as thymidine glycols (dTG)[1] and (hydroxymethyl)deoxyuridine (dHMU) have been quantitated following the irradiation of cultured cell lines (Cerutti, 1976; Frenkel et al., 1981b, 1985; Teebor et al., 1982, 1984). 5,6-Dihydrothymidine (dDHT), a product of the anoxic irradiation of thymidine, has been reported to occur in irradiated DNA (Teoule et al., 1978; Dizdaroglu, 1985). However, the exact amount of dDHT formed under various conditions and its production in irradiated cells has not been reported. Here we report a method for the quantitation of the radiation product dDHT in ^{3}H-labeled DNA. dDHT is quantitated by HPLC following enzymatic digestion of the DNA to deoxynucleosides. The method has been used to quantitate each of the two stereoisomers of dDHT (Figure 1) in DNA following γ-irradiation under a variety of irradiation conditions or following γ-irradiation of intact cultured cells. It will be demonstrated that dDHT production occurs at substantial levels only under strictly anoxic conditions and that, in DNA, the *R* stereoisomer is formed preferentially.

Experimental Procedures

Materials. Thymine, thymidine, 5-(hydroxymethyl)uracil, 5-(hydroxymethyl)deoxyuridine, 5,6-dihydrothymine, and 5,6-dihydrothymidine were obtained from Sigma Chemical Co., St. Louis, MO. [*methyl*-^{3}H]thymidine (76 Ci/mmol) and [6-^{3}H]thymidine (18.2 Ci/mmol) were obtained from New England Nuclear (Boston, MA). All solvents were HPLC grade (Burdick and Jackson, Muskegon, MI).

Preparation of dTG, TG, (R)-dDHT, and (S)-dDHT. *cis*-5,6-Dihydroxy-5,6-dihydrothymine and *cis*-5,6-dihydroxy-5,6-dihydrothymidine were prepared according to the method of Iida and Hayatsu (1970, 1971) as modified by

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[1] Abbreviations: dDHT, 5,6-dihydrothymidine; dTG, 5,6-dihydroxy-5,6-dihydrothymidine; DHT, 5,6-dihydrothymine; TG, 5,6-dihydroxy-5,6-dihydrothymine; dHMU, 5-(hydroxymethyl)-2′-deoxyuridine; HMU, 5-(hydroxymethyl)uracil; HPLC, high-pressure liquid chromatography; dT, thymidine; T, thymine.

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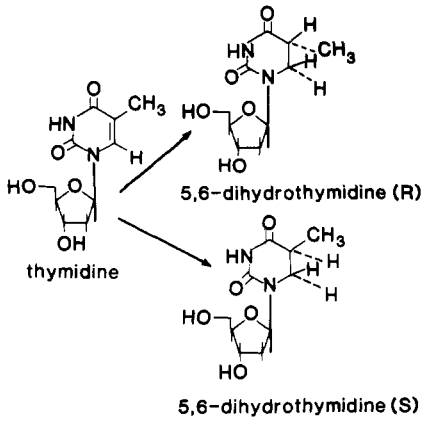

FIGURE 1: Structures of thymidine and the two stereoisomers of 5,6-dihydrothymidine.

Frenkel et al. (1981a,b). Purified (*R*)-dDHT and (*S*)-dDHT were prepared from the mixture of the two isomers in commercial dDHT by chromatography on reverse-phase HPLC as described below. Purity (>95%) was assessed by a second run on normal-phase HPLC. The identity of the two compounds purified from commercial dDHT as authentic isomers of dDHT was established by fast atom bombardment mass spectroscopy. Both of the compounds produced the same protonated molecular ion peak at *m/z* 245 and fragments at *m/z* 129 (base + 2 H) and *m/z* 117 (deoxyribose). The melting points of the *R* and *S* isomers were determined to be 124–125 °C and 160 °C (dec), respectively. The identity of (*R*)-dDHT and (*S*)-dDHT was established by their relative retention times on a reverse-phase HPLC system described by Cadet and co-workers (Cadet et al., 1981).

Cell Culture, [^{3}H]Thymidine Radiolabeling, and DNA Isolation. HeLa cells were grown as monolayers in DME medium supplemented with 10% fetal calf serum at 37 °C and in a 5% CO_2 atmosphere. To cells at 50% confluency (6×10^6 cells) in a 150-cm^2 flask containing 50 mL of media was added 0.5 mCi of ^{3}H-labeled thymidine. Following 24-h further incubation, the media were removed and the cells harvested by trypsinization. Suspended cells were washed with phosphate-buffered saline and resuspended in the same solution.

DNA was isolated from either irradiated or unirradiated HeLa cells as described by Grunberg and Haseltine (1980), with further purification from residual RNA as described by Franklin and Haseltine (1983). DNA concentrations were determined by the absorbance at 260 nm (A_{260} of 22 = 1 mg/mL). Specific activities of the purified DNA ranged from 2.5×10^5 to 7.9×10^5 cpm/μg.

γ-Irradiation. Cobalt-60 γ-rays were delivered by a Gammarad irradiator, Model GR9, at a dose rate of 0.45 Gy/s. Samples were purged with either O_2 or oxygen-free N_2 for 10 min prior to and during the irradiation. Measurement of the outflow gas from samples purged with oxygen-free N_2 detected <6 ppm oxygen (Thermox TM1A oxygen meter). Where indicated, samples were purged with an intermediate concentration of oxygen (73 ppm) created by mixture of a small amount of air into the flow of oxygen-free nitrogen. Unless otherwise noted, irradiation was performed at ambient temperature.

Within 72 h prior to irradiation, ^{3}H-labeled thymidine was subjected to "cleanup" on reverse-phase HPLC and then stored in methanol and −20 °C until used. Samples of thymidine for irradiation were lyophilized and then dissolved at a concentration of 0.1 mM in 10 mM sodium phosphate (pH 7.5) either with or without 100 mM potassium iodide. Samples of DNA for irradiation were extensively dialyzed against the same buffer and adjusted to a final concentration of 0.1 mM (as nucleotide). Single-stranded DNA was prepared immediately before irradiation by heating samples of DNA in irradiation buffer to 95 °C for 5 min, followed by quick cooling. HeLa cells to be irradiated were suspended at a concentration of 6×10^5 cells/mL in phosphate-buffered saline and irradiated at 0 °C.

Digestion of DNA to Deoxynucleosides. Samples of irradiated or unirradiated control ^{3}H-labeled DNA (3.3 μg) were ethanol-precipitated and lyophilized. Authentic (*R*)-dDHT and (*S*)-dDHT were added as internal controls. The DNA was then digested to 3′-deoxynucleotides as described by Franklin and Haseltine (1983). To the digest (40 μL) was added 0.03 unit of calf alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN), and incubation was continued at 37 °C for 1 h. Additional phosphatase was added and the incubation repeated until digestion to 3′-deoxynucleosides was completed. Completion of digestion was determined by elution from PEI–cellulose. Deoxynucleosides [including dT, (*R*)-dDHT, (*S*)-dDHT, and dHMDU] are completely removed from PEI–cellulose by methanol while DNA, oligonucleotides, and mononucleotides are retained. Duplicate samples (2 μL) of each sample were spotted onto a piece of PEI–cellulose TLC plate (Brinkmann Instruments, Westbury, NY) and dried. One of the two aliquots was rinsed with methanol and dried. Both pieces of TLC plate were then placed into liquid scintillation fluid, and radioactivity was determined. The fraction of DNA not digested to deoxynucleosides is the radioactivity on the methanol-washed plate divided by the radioactivity on the unwashed plate. If digestion was >80% complete, the entire sample was spotted onto PEI–cellulose and eluted with methanol. The eluate was lyophilized to dryness and resuspended in HPLC mobile-phase buffer.

HPLC. HPLC equipment included a Spectra-Physics SP8700 solvent delivery system and a Rheodyne injector. Detection was achieved with an ISCO V4 detector set at 220 nm. Flow rate was 1.0 mL/min. HPLC system I included a μBondapak C_{18} column (30 × 0.39 cm, Waters Associates, Milford, MA) and a reverse-phase guard column (Alltech Associates, Deerfield, IL) and a mobile phase of water–methanol (19:1 v/v). Retention times (in min) of marker compounds were as follows: 3.9, TG; 4.7, HMU; 5.8, dTG; 7.2, DHT; 8.3, T; 8.7, dHMU; 9.7, (*R*)-dDHT; 11.4, (*S*)-dDHT; 17.6 dT. HPLC system II included a 605SI silica column (5 μm, 25 × 0.46 cm, Alltech Assoc.) and a mobile phase of heptane–2-propanol (17:3 v/v). Retention times (min) of marker compounds were as follows: 9.6, T; 13.2, HMU; 13.7, dT; 14.5, DHT; 17.4, dHMU; 19.2, (*S*)-dDHT; 21.9 min, (*R*)-dDHT. On HPLC system II, the retention times for all the above compounds decreased as the silica column aged. However, the order of elution did not change, and the resolution of the various compounds was retained.

Calculation of G Values. A G unit represents one molecule of product formed per 100 eV of energy absorbed. In aqueous solution a G unit is equivalent to 1.04 μM product per 10 Gy of absorbed dose (Hutterman et al., 1978). Therefore, for samples irradiated at 0.1 mM

$$G = 961(F/D)$$

where *G* is the *G* value for product, *F* is the fraction of total radioactivity recovered as product, and *D* is the dose in Gy. Each *G* value was calculated from irradiation at multiple doses (usually 3), and then the mean and the standard deviation were determined.

Table I: *G* Values for γ-Irradiation Production of 5,6-Dihydrothymidine[a]

substrate	expt	irradiation conditions: atm N_2	atm O_2	KI[b]	*G* value (×10³) (±SD): (*R*)-dDHT	(*S*)-dDHT
thymidine	1	+			248 ± 57	272 ± 78
	1		+		7.9	12 ± 2.8
	2	+		+	2.4 ± 0.26	2.4 ± 0.44
	2		+	+	0.24 ± 0.074	0.51 ± 0.33
DNA						
native	3	+			19.9 ± 3.3	11.3 ± 3.2
	5	+			10.5 ± 1.8	5.9 ± 0.55
	6[c]	+			20.4 ± 5.3	10.6 ± 3.4
	7[c]	+			14.6 ± 0.6	11.3 ± 0.4
	4		+		<0.4	<0.4
	5		+		<0.4	<0.4
	7[c]		+		<0.4	<0.4
	7[c]	+		+	<0.4	<0.4
	7[c]		+	+	<0.4	<0.4
denatured	5	+			4.9 ± 0.15	4.0 ± 0.09
	7[c]	+			2.2 ± 0.13	1.8 ± 0.25
cellular	8	+			104	<30
	9[c]	+			116 ± 26	<30

[a] γ-Irradiation (0–1600 Gy) and quantitation of (*R*)- and (*S*)-5,6-dihydrothymidine were performed as described under Experimental Procedures. [b] Indicated samples were irradiated in the presence of 100 mM KI. [c] In experiments 6, 7, and 9, DNA was labeled by incorporation of [6-³H]thymidine. In experiments 1–5 and 8, DNA was labeled with [methyl-³H]thymidine.

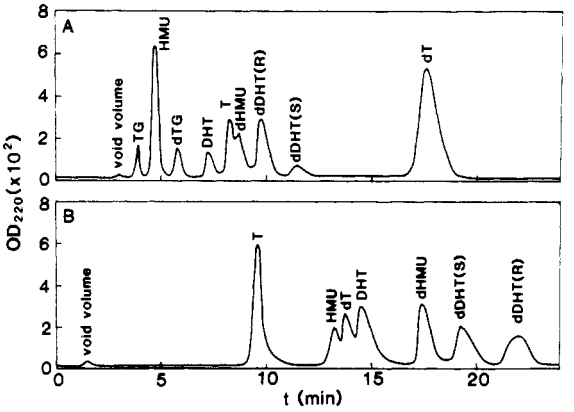

FIGURE 2: HPLC resolution of some products of γ-irradiation of thymidine. Mixtures containing various amounts of T, TG, dTG, HMU, dHMU, (*R*)-dDHT, (*S*)-dDHT, and dT were resolved on two HPLC systems as described under Experimental Procedures. (A) HPLC system I; (B) HPLC system II.

RESULTS

Assay System. Reverse-phase HPLC, using system I described under Experimental Procedures, provides excellent resolution of several of the known products of thymidine irradiation (Cadet et al., 1982; De Abreu et al., 1982; Gehrke et al., 1980). The stereoisomers (*R*)-dDHT and (*S*)-dDHT elute at 9.7 and 11.4 min, respectively (Figure 2A), well resolved from each other and from T, TG, HMU, dT, dTG, and dHMU. In the experiments described below, reverse-phase HPLC (system I) was selected for quantitation of dDHT because of its convenience and high resolution and because the absolute retention times remained constant over several months of column use. In quantitating the amount of dDHT produced by irradiation, the identity of the radioactive product eluting at the position of either (*R*)-dDHT or (*S*)-dDHT was confirmed by a second chromatography on normal-phase HPLC (Figure 2B). On normal-phase chromatography, the order of elution of the *R* and *S* isomers is reversed, and the *R* isomer is completely resolved from any contamination by dHMU. Normal-phase HPLC (system II) was less useful for routine quantitation of dDHT, as the absolute retention times of dDHT and other thymine derivatives vary with column age and are relatively sensitive to small changes in mobile phase.

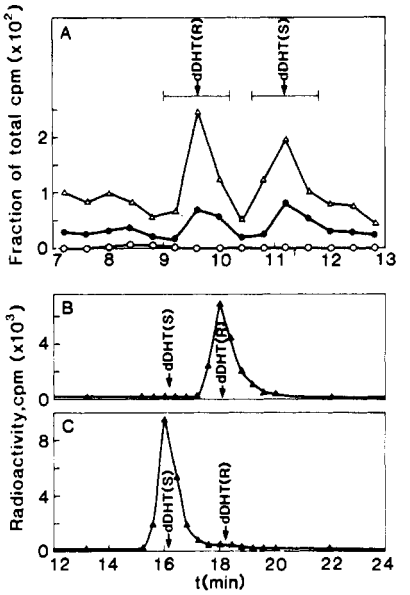

FIGURE 3: Production of dDHT isomers by γ-irradiation of thymidine. [³H]TdR was γ-irradiated to a dose of 0 (○), 50 (●), or 200 Gy (△) under nitrogen as described under Experimental Procedures. (A) (*R*)-dDHT and (*S*)-dDHT were added and 6.5 × 10⁵ cpm of each sample were resolved on HPLC system I. Fractions were collected each 0.4 min, and radioactivity was determined. The remainder of fractions at the times indicated by the horizontal bars were pooled, lyophilized, and resolved on HPLC system II. Fractions were collected as indicated, and radioactivity was determined. Panels B and C are the second chromatography of (*R*)-dDHT and (*S*)-dDHT, respectively.

Production of dDHT by γ-Irradiation of Thymidine. The production of dDHT by γ-irradiation of [*methyl*-³H]thymidine was determined with this system. Neither of the dDHT isomers was detectable in the absence of γ-irradiation. Following γ-irradiation of thymidine (0.1 mM) under nitrogen, radioactive products eluted at the positions of (*R*)-dDHT and (*S*)-dDHT (Figure 3A). The increase in radioactivity eluted at these positions was linearly related to dose up to 200 Gy. The identification of these two radiation products as (*R*)-dDHT and (*S*)-dDHT was confirmed by analysis on HPLC system II (Figure 3B,C). The amount of dDHT produced from thymidine by γ-rays under various irradiation conditions is shown in Table I. dDHT is a major product of thymidine

irradiation under strictly anoxic conditions and in the absence of free-radical scavenger. The presence of oxygen during irradiation drastically reduces production of dDHT (~30-fold). The presence of the free-radical scavenger potassium iodide during irradiation also markedly reduces dDHT production. Irradiation of thymidine produces approximately equal amounts of the two stereoisomers (*R*)-dDHT and (*S*)-dDHT.

Production of dDHT by γ-Irradiation of DNA in Vitro. To determine the amount of dDHT in DNA following γ-irradiation, ^{3}H-labeled native DNA from HeLa cells was isolated, irradiated (0–1600 Gy), and digested to deoxynucleosides. Residual amounts of nucleotides and polynucleotides were removed by adsorption to PEI–cellulose and elution of deoxynucleosides with methanol. The deoxynucleoside mixture was then resolved on HPLC system I as described above. For DNA irradiated under anoxia, peaks of radioactivity elute at the positions of both (*R*)-dDHT and (*S*)-dDHT. Rechromatography of pooled samples coeluting with (*R*)-dDHT and (*S*)-dDHT markers on HPLC system II established the identity of the material eluting at these positions as authentic (*R*)-dDHT and (*S*)-dDHT, respectively.

The amount of dDHT produced in DNA irradiated in vitro under various conditions is shown in Table I. The *G* value for production of dDHT from DNA is less (10–15-fold) than the *G* value for production of dDHT from thymidine. When irradiation was performed in the presence of either oxygen or potassium iodide, dDHT could not be detected in digests of DNA. When DNA samples were irradiated under an atmosphere containing an intermediate concentration of oxygen (73 ppm), rather than under strictly anoxic conditions (<6 ppm oxygen), dDHT formation was detected, but the yield was reduced approximately 5-fold for both the (*R*)-dDHT (*G* value 0.0029) and (*S*)-dDHT (*G* value 0.0021) stereoisomers.

Production of dDHT by irradiation of DNA differs quantitatively from irradiation of thymidine in two ways. The total amount of dDHT produced is less when DNA is irradiated. Also, on irradiation of DNA, significantly less (*S*)-dDHT than (*R*)-dDHT is produced ($p < 0.01$). Inspection of Table I reveals that consistently more (*R*)-dDHT than (*S*)-dDHT was produced by a factor of 1.71 ± 0.32. Because no such preferential production of (*R*)-dDHT was observed on irradiation of thymidine, we hypothesized that stereospecificity of production of dDHT in DNA might be imparted by the conformation of double-stranded DNA. To test this possibility, DNA was irradiated following heat denaturation and the amount of the two stereoisomers quantitated as above. When denatured, the amount of (*S*)-dDHT was again less than that of (*R*)-dDHT but the ratio of (*R*)-dDHT to (*S*)-dDHT more closely approached unity (1.21 ± 0.06). The lower ratio of 1.21 for single-stranded DNA compared to 1.71 for double-stranded DNA was significant ($p < 0.005$).

Control experiments in which DNA was spiked with known amounts of the two dDHT isomers have eliminated the possibility that either the reduction of *G* value when DNA is the irradiation substrate or the ratio of (*R*)-dDHT to (*S*)-dDHT observed is an artifact due to selective loss of (*R*)-dDHT or (*S*)-dDHT during the digestion and cleanup steps prior to HPLC resolution.

Other investigators (Teebor et al., 1982, 1984) have reported that decay of incorporated tritium can produce radiation products in DNA. In particular, decay of ^{3}H label at the methyl position of thymidine produces dHMU with high frequency. To determine whether the apparent yields of dDHT were affected by the position of the ^{3}H label, DNA was prepared from cells labeled with tritium at either the methyl or 6-position of thymidine. Inspection of Table I shows that the position of the label does not alter the *G* values obtained.

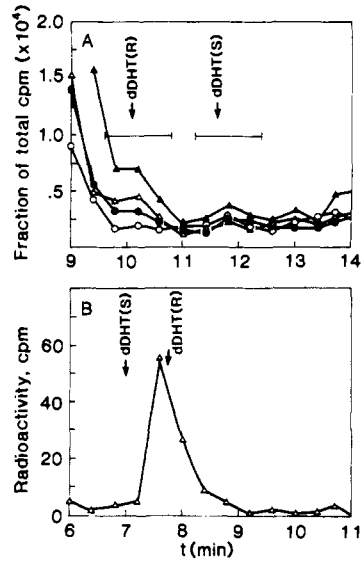

FIGURE 4: Production of (*R*)-dDHT in DNA of γ-irradiated HeLa cells. HeLa cells were labeled with [6-^{3}H]thymidine, γ-irradiated under nitrogen as described under Experimental Procedures. DNA was isolated from cells irradiated to 0 (○), 500 (●), 1000 (△), and 2000 Gy (▲), digested to deoxynucleosides, and resolved on HPLC system I. (A) For each sample, 1.1×10^6 cpm was injected. Fractions were collected, and radioactivity was determined as described in Figure 3A. (B) Fractions of (*R*)-dDHT at the position of the horizontal bar in panel A were pooled and resolved by HPLC system II.

Production of dDHT in DNA of Irradiated Cultured Cells. To determine whether dDHT is a product of γ-irradiation of living cells, the DNA of cultured human cells (HeLa) was labeled by incorporation of [^{3}H]thymidine, and the cells were irradiated (0–2000 Gy) under anoxia. Following irradiation, the cells were immediately lysed and the [^{3}H]thymidine-labeled DNA was isolated, digested to deoxynucleosides, and analyzed by HPLC system I as described above. The results of one such experiment are shown in Figure 4A. DNA from irradiated cells contains increased amounts of radioactivity eluting at the position of (*R*)-dDHT. Material eluting in these fractions was positively identified as (*R*)-dDHT by a second chromatography on HPLC system II (Figure 4B). In some samples of DNA from irradiated cells, there was also an increase in radioactivity eluting at the position of (*S*)-dDHT. However, the amount of radioactivity at this position was not clearly dependent on radiation dose. Attempts to positively identify (*S*)-dDHT in irradiated samples were unsuccessful as <8% of the radioactivity eluting at the position of (*S*)-dDHT on HPLC system I was recovered as (*S*)-dDHT during a second chromatography on HPLC system II. We conclude from these results that γ-irradiation under anoxic conditions forms $(0.62 \pm 0.2) \times 10^{-7}$ mol of (*R*)-dDHT per mole of cellular DNA (as nucleotide) per gray. Production of (*S*)-dDHT was not detected by this method and is $<0.2 \times 10^{-7}$ mol per mole of DNA nucleotides per gray. Thus, dDHT is produced in cellular DNA during irradiation under anoxia, and the (*R*)-dDHT stereoisomer is preferentially produced with a ratio of (*R*)-dDHT to (*S*)-dDHT at least as great as was observed following irradiation of isolated double-stranded DNA in vitro.

The *G* value for formation of (*R*)-dDHT in the nuclei of HeLa cells was calculated with the same assumptions as those of Frenkel and her co-workers (Frenkel et al., 1985): an average DNA content of 16×10^{-12} g/HeLa cell nucleus and

an average nuclear radius of 4 μm with a density of 1. The G value for formation of (R)-dDHT in cellular DNA calculated in this way is 0.11.

Discussion

In recent years, there have been several reports of the use of HPLC technology for the quantification of altered deoxynucleosides in DNA following γ-irradiation. HPLC resolution of deoxynucleosides from digested DNA has been used to quantitate dTG and dHMU in irradiated DNA (Frenkel et al., 1981a,b, 1985; Teebor et al., 1982, 1984). The use of HPLC following digestion of DNA to deoxynucleosides allows both sensitive and specific detection of radiation-altered bases. This paper demonstrates an HPLC method suitable for the quantification of the radiation product dDHT in DNA following γ-irradiation of DNA either in vitro or in cultured human cells. An additional advantage of this method of measuring dDHT in DNA was the ability to quantitate the amounts of individual R and S isomers. While the presence of dDHT in DNA, γ-irradiated in vitro, has been reported (Teoule et al., 1978; Dizdaroglu, 1985), neither the amounts produced nor the presence of this lesion in the DNA of irradiated cells has been reported. The ability to quantitate these specific lesions in DNA provides a first step in studies of their possible biological significance.

In solutions of thymidine, γ-irradiated under anoxia, dDHT is a major product (G value ~0.5). This G value is somewhat greater than the G value of 0.1 previously reported by others (Cadet, 1973; Cadet et al., 1981). Because of the marked inhibition of dDHT production that occurs in the presence of oxygen, we attribute the greater G value for dDHT production obtained in our experiments to the strictly anoxic conditions of irradiation rather than to differences in the method of quantitation. The reaction sequence that produces dDHT is believed to be initiated by a solvated electron (Teoule et al., 1978). Such electrons are converted to the much less reactive $O_2^{\cdot -}$ in the presence of molecular oxygen (Scholes, 1978), and this may explain the marked sensitivity of dDHT production to oxygen.

The amount of dDHT produced by γ-irradiation of DNA is considerably less than that produced by irradiation of thymidine. This reduction in dDHT production in DNA may be due either to the conformation of thymidine when incorporated into DNA or to competition by other components of DNA for the reactive species. Oxygen also markedly inhibits the production of dDHT in DNA.

The G value for formation of (R)-dDHT in cellular DNA is higher than the G value for formation in DNA in solution. This result is contrary to the general assumption that formation of radiation products due to indirect action of ionizing radiation will be much reduced in the cell due to the presence of high concentrations of free-radical scavengers. However, Frenkel and her co-workers have recently reported that the G value for formation of dHMU is substantially higher with cell DNA as a target than when DNA in solution is irradiated. The reason for such an increased yield in the nuclear environment is not understood.

In agreement with the results of Cadet (1973), we find that γ-irradiation of thymidine produces equal amounts of (R)-dDHT and (S)-dDHT. However, irradiation of DNA produces consistently more (R)-dDHT than (S)-dDHT. This stereospecificity of the reaction producing dDHT in DNA is likely to be due to the conformation of thymidine in the DNA double helix. Further evidence consistent with this interpretation is our observation that the ratio of (R)-dDHT to (S)-dDHT approaches unity when DNA is irradiated in the less ordered, single-stranded form. In addition, the results reported above show that the formation of (R)-dDHT is favored in the cellular DNA.

As yet, little is known of the biochemical consequences of dDHT in DNA. As for dTG, the base residue of dDHT has lost planarity and should disrupt the local conformation of the DNA double helix. Both dTG and dDHT are recognized and removed from DNA by the glycosylase action of *Escherichia coli* endonuclease III (Demple & Linn, 1980) and the *Micrococcus luteus* γ-endonuclease (T. J. Jorgensen, unpublished data). The common feature to these lesions would appear to be loss of planarity of the thymine base. The presence of dTG in DNA has also been shown to provide "stop" sites for DNA polymerase (Rouet & Essigmann, 1985; Ide et al., 1986; Clark & Beardsley, 1986). Whether dDHT will provide similar blocks to polymerization will be of interest.

The biological significance of dDHT as a radiation product in mammalian cells is also not known. The effect of oxygen on radiation-induced mutagenesis is not clear, and dDHT may represent a significant mutagenic lesion for mammalian cells irradiated under hypoxia. There is no direct evidence, however, to support this speculation at present. The anoxic conditions under which dDHT is most abundantly produced have long been known to be radioprotective for cell killing (Mottram, 1936; Read, 1952; Gray et al., 1953). This suggests that dDHT is not the major lethal lesion produced by γ-irradiation except possibly under very hypoxic conditions.

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Registry No. *cis*-dTG, 42957-13-1; (R)-dDHT, 78216-59-8; (S)-dDHT, 19140-39-7; *cis*-5,6-dihydroxy-5,6-dihydrothymine, 1124-84-1.

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Nucleotide Sequence Binding Preferences of Nogalamycin Investigated by DNase I Footprinting†

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ABSTRACT: Four DNA restriction fragments, designated *tyrT*, *pTyr2*, *pUC13*, and *Xbs1*, have been used as substrates for footprinting studies with DNase I in the presence of the anthracycline antibiotic nogalamycin. With each fragment a distinct pattern of antibiotic-protected binding sites is observed, but no concensus sequence emerges from the data. All sites are located in regions of alternating purine–pyrimidine sequence, most commonly associated with the dinucleotide steps TpG (CpA) and GpT (ApC), suggesting that the preferred binding sites may contain all four nucleotides and/or that peculiarities of the dynamics of DNA conformation at alternating sequences may be critical for nogalamycin binding. Some concentration dependence of footprinting patterns is evident, in contrast to previous studies with a variety of sequence-specific ligands. Enhanced susceptibility to attack by DNase I is commonly observed at sequences flanking strong antibiotic-binding sites. Nogalamycin selectively inhibits cleavage of DNA at certain guanine-containing sequences by the G-specific photosensitized reaction with methylene blue. Comparison of these effects with its action on the G-specific reaction with dimethyl sulfate suggests that the amino sugar moiety of nogalamycin may be preferentially located in the minor helical groove at some binding sites but in the major groove at others.

Nogalamycin (Figure 1) is an anthracycline antibiotic that has been reported to show activity against experimental tumors both in vivo and in vitro (Bhuyan & Deitz, 1965; Bhuyan & Reusser, 1970; Brown, 1983). Most anthracyclines bear a sugar substituent at position 7 on the nonaromatic A ring of the chromophore; others (such as iremycin) have one on the opposite side of the A ring at position 10 (Arcamone, 1981; Ihn et al., 1980). The structure of nogalamycin is unique insofar as it bears bulky sugar residues at both ends of its anthracycline chromophore (Arora, 1983). Nevertheless, it retains the property of binding strongly to double-helical DNA by the well-investigated mechanism of intercalation (Kersten et al., 1966; Waring, 1970; Plumbridge & Brown, 1979). The current model proposed for its interaction with DNA involves positioning of the two sugar moieties in each of the helical grooves, with the chromophore "spearing" the stack of base pairs (Arora, 1983; Collier et al., 1984; Fox & Waring, 1984a). While this model is sterically feasible, it poses considerable dynamic problems since the minimum width of the antibiotic is 1.2 nm, whereas it is not possible to open a potential DNA intercalation site beyond about 1.0 nm (Collier et al., 1984). We have previously shown that an important consequence of this limitation is apparent in the kinetics of antibiotic binding, which are markedly dependent upon the precise nucleotide sequence. The antibiotic binds fastest to those sequences that are easier to disrupt (Fox & Waring, 1984) yet dissociates most slowly from the more stable regions (Fox et al., 1985).

In all these respects, nogalamycin behaves very differently from such simple anthracyclines as daunomycin. On the basis of our kinetic findings, we predicted that nogalamycin might display some peculiar sequence preferences, binding best to those regions of DNA that are most easily disrupted (melted) yet that can be most strongly stabilized by the presence of the antibiotic. Previous investigations aimed at detecting possible base sequence selectivity in the binding of this antibiotic to

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