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QUANTITATIVE MEASUREMENT OF THE DIASTEREOISOMERS OF *CIS* THYMIDINE GLYCOL IN GAMMA-IRRADIATED DNA

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A technique for determing the relative content of each of the diastereoisomers of *cis* thymidine glycol (dTG) in DNA exposed to ionizing radiation has been developed. [^{3}H]thymidine DNA was gamma-irradiated, digested to 2′-deoxyribonucleosides, authentic [^{14}C] (+, −) *cis* dTG added to the digestate and the mixture resolved by HPLC. ^{3}H fractions coeluting with [^{14}C] (+, −) dTG were collected and acetylated.

The acetoxy derivatives of (+) and (−) *cis* dTG were easily resolved by a second HPLC analysis and their absolute configuration determined by NMR amd mass spectroscopies. We have constructed a dose-response curve for formation of each isomer in gamma-irradiated DNA and shown that they are formed in equal amounts. This technique may be used to determine the relative formation of *cis* dTG isomers in DNA resulting from other oxidative stresses and whether repair of these is influenced by their configuration.

KEY WORDS: *cis* Thymidine glycol, diastereoisomers, acetylation, DNA, gamma-irradiation, oxidative damage.

INTRODUCTION

Of the bases in DNA, thymine is the most susceptible to modification by oxidative stress[1] and ionizing radiation.[2] The oxidative derivative most readily formed is the *cis* 5,6 dihydroxy-5,6-dihydrothymine (thymine glycol, TG) moiety whose radiogenic formation in DNA has been documented.[3,4] However, the *cis* TG nucleoside moiety (dTG) consists of a diastereoisomeric pair (+, −)[5] whose relative radiogenic formation has never been measured. This measurement would be of some interest for it has been reported that 10 times more of one dTG stereoisomer is excreted in mammalian urine than the other.[6] It has been proposed that this finding is indicative of selective formation and/or repair of one diastereoisomer. Techniques previously used for measuring the dTG content of solutions of DNA exposed to radiation have not been able to measure the relative quantity of isomer formed, for the *cis* dTG diastereoisomers are not readily separable by liquid chromatographic means.[7] To effect this separation we subjected the (+, −) dTG mixture to acetylation thereby forming their acetoxy derivatives. These proved to be readily separable by HPLC and we were therefore able to measure the relative formation of the dTG diastereoisomers in gamma-irradiated DNA.

MATERIALS AND METHODS

A. Marker Compounds [^{14}C] *cis* dTG (+, −) were obtained by $KMnO_4$ oxidation of [^{14}C] thymidine and subsequent purification by HPLC.[4,8] The final specific activity of the authentic marker was approximately 150 μCi/mmole.

B. Acetylation For analytic purposes, approximately 10^5 cpm of ^{14}C (10 μg) was dissolved in 500 μl of dry, redistilled pyridine and 600 μl of acetic anhydride at 25°C overnight (16 h). 5 ml of H_2O were added, the volume reduced by vacuum evaporation and the residue injected in a volume of 200 μl into an ODS 25 × 1.4 cm column and eluted with acetonitrile-H_2O (30/70) at a flow rate of 2 ml/min. One ml fractions were collected and counted by liquid scintillation counting.

For NMR and mass spectroscopic analysis 100 mg of purified non radioactive dTG (+, −) was acetylated and the HPLC peaks monitored by 254 nm absorption. Peaks corresponding to ^{14}C retention times were collected, dried and analyzed.

C. Mass Spectroscopic and NMR Analysis Fast atom bombardment (FAB) mass spectra were obtained by using a Kratos spectrometer model MS 50 equipped with a commercially available FAB gun. Desorption of the molecules from a glycerol mull was obtained by exposure to a 8 keV beam of xenon atoms. The 250 MHz ^{1}H Nuclear magnetic resonance spectra were obtained on a Bruker WM 250 spectrometer. Tetradeurated methanol was used as the solvent with tetramethylsilane as the internal reference.

D. Preparation of [^{3}H] thymidine DNA An *E. coli thy*(−)strain was grown in medium containing [^{3}H-methyl]thymidine and the DNA purified as previously described.[4,8] The final specific activity of the DNA was 20–50,000 cpm/μg as measured by absorbance at 260 nm assuming $1A_{260} = 50\,\mu g$.

E. Irradiation DNA, either double stranded (ds) or single stranded (ss) (boiling water bath 10 minutes) was irradiated in aerated, double glass distilled H_2O from a ^{137}Cs source at a dose rate of 4.5 Gy/min. The concentration of the DNA was 25 μg/ml.[4,9]

F. Enzymatic Digestion of irradiated [^{3}H] DNA The DNA was exposed successively to DNAse I, snake and spleen phosphodiesterases and alkaline phosphatase yielding 2′-deoxyribonucleosides. After overnight digestion the mixture was treated with 5 volumes of cold acetone, centrifuged, the supernatant volume reduced and the entire sample analyzed by HPLC.[4,9]

G. HPLC analysis Approximately 2 × 10^6 cpm of ^{3}H activity representing the supernatant of the acetone precipitated digestate were analyzed for each radiation point (100–200 μg DNA total) together with 10^4 cpm of authentic [^{14}C]dTG (+, −) as previously described.[4,8] The fraction coeluting with the [^{14}C] marker was collected, dried, acetylated under analytical conditions and reanalyzed by HPLC.

H. Determination of the relative and absolute content of the cis dTG (+) and (−) diastereoisomers in gamma-irradiated DNA The ^{14}C peaks were counted and the

yield of recovered acetate determined. This was generally 40–60%. The ^{3}H content coeluting with each ^{14}C peak was determined, the yield of acetylation assumed to be the same as for ^{14}C and the number of ^{3}H cpm recovered divided by the total ^{3}H injected into the first HPLC column × 100. This yielded % formation of (+) and (−) *cis* dTG.

RESULTS

Figure 1 shows a representative chromatographic profile of the mixture of [^{14}C] (+, −) dTG and ^{3}H-material coeluting with [^{14}C] dTG after acetylation as analysed by HPLC. Four ^{14}C peaks were obtained which were labelled A, B, a, b. It may be noted that there is approximately twice as much ^{14}C radioactivity in B and b as compared with A and a. This was a consistent finding in acetylation of (+, −) dTG prepared by $KMnO_4$ oxidation of thymidine and will be discussed below. In contrast, the ^{3}H content of the peaks is about the same. This is the DNA-derived material.

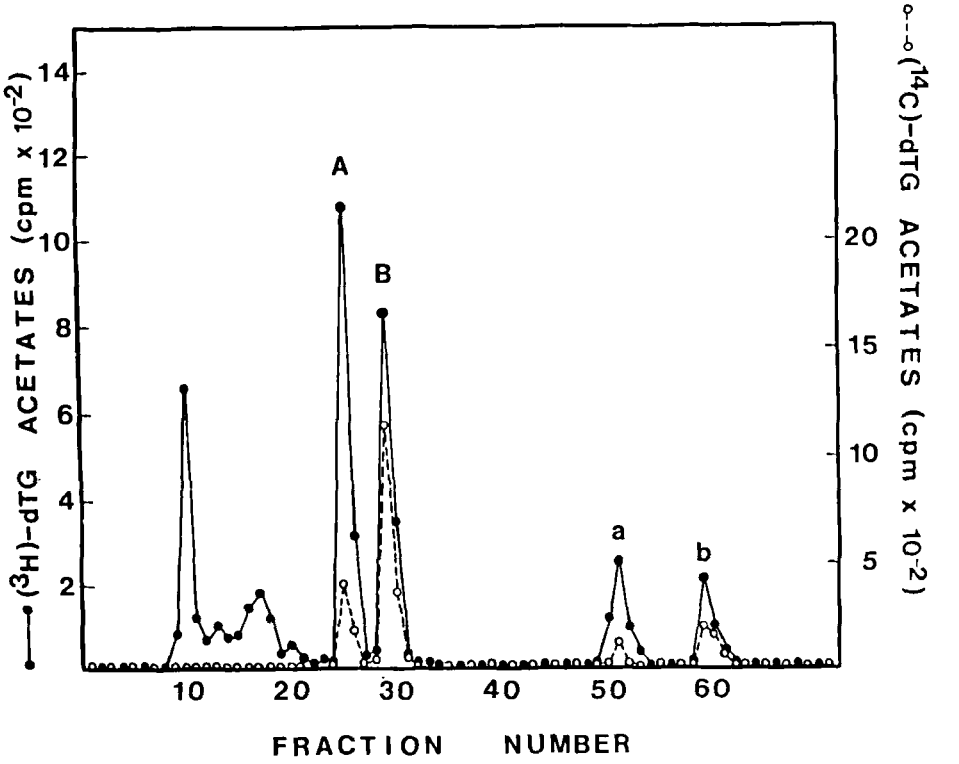

FIGURE 1 HPLC analysis of the acetylated (+, −) *cis* dTG fraction which was obtained from the digestate of [^{3}H] thymidine labelled DNA exposed to gamma radiation. Peaks A and a and B and b are the tri- and tetra-acetoxy derivatives of the (+) and (−) *cis* dTG stereoisomers respectively. The ^{14}C content is derived from dTG made by chemical ($KMnO_4$) oxidation of [^{14}C] thymidine. The ^{3}H content represents radiogenically formed dTG. Analysis was with an ODS 25 × 1.0 cm column, acetonitrile-H_2O (7:3). Flow rate 2 ml/minute.

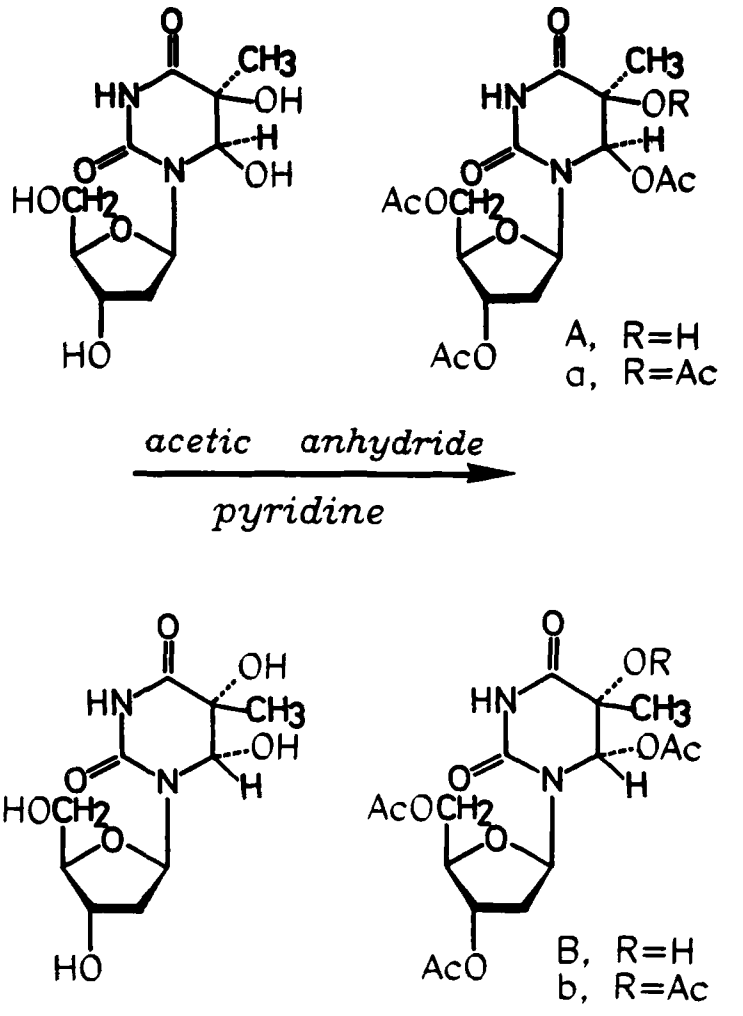

FIGURE 2 Chemical structures of the tri-O and tetra-O-acetyl derivatives of (+) *cis* (5S, 6R) and (−) *cis* (5R, 6S)-5,6-dihydroxy-5,6-dihydrothymidine.

The radiation-induced degradation products of [^{3}H] thymidine obtained after enzymatic hydrolysis of DNA, followed by HPLC separation, subsequent acetylation and HPLC reanalysis have been characterized by comparison of their FAB-MS and ^{1}H NMR features with those of authentic samples. Observation of a pseudomolecular ion at m/z 425 $(M + Na)^+$ in the positive mode FAB-MS spectrum of A and B nucleosides is consistent with the structure of a tri-O-acetylated derivative of thymidine glycol. Further support for the assignment of these modified nucleosides was provided by ^{1}H NMR analysis (Table 1). Three acetoxy groups are observed in the 250 MHz ^{1}H NMR spectra of compounds A and B. In addition the position of the acetyl functions is given by the expected downfield shift effects (0.6–1.2 ppm) for a methine proton when the acetoxy group is in an α position. Therefore A and B

TABLE I
Chemical shift values (δ) of the (+) and (−) *cis* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine and their corresponding tri-O- and tetra-O-acetylated derivatives[a]

Nucleosides	CH_3	H-6	H-3′	H-5′	H-5″
(+) *cis* (5S, 6R)-5,6-dihydroxy-5,6-dihydrothymidine	1.38	4.91	4.36	3.74	3.68
tri-O-acetyl derivative A	1.46	6.17	5.15	4.18	4.14
tetra-O-acetyl derivative a	1.81	6.71	5.17	4.19	4.14
(−) *cis* (5R, 6S)-5,6-dihydroxy-5,6-dihydrothymidine	1.38	4.99	4.32	3.68	3.66
tri-O-acetyl derivative B	1.42	6.20	5.19	4.33	4.28
tetra-O-acetyl derivative b	1.77	6.79	5.17	4.29	4.24

[a]Proton data at 250 MHz in ppm from tetramethylsilane used as internal reference in CD_3OD.

degradation products have been identified as the tri-O-3′,5′,6-acetoxy derivatives of (+) *cis* (5S, 6R)-5,6-dihydroxy-5,6-dihydrothymidine and (−) *cis* (5R, 6S)-5,6-dihydroxy-5,6-dihydro-thymidine respectively. Compounds a and b have been characterized as the corresponding tetra-O-acetyl-3′,5′,5,6-derivatives.

DISCUSSION

The quantitative acetylation of the (+) and (−) *cis* dTG diastereoisomers and their facile separation by HPLC has permitted the accurate measurement of their formation in gamma-irradiated DNA. These results indicate that there is apparently no selective formation of one or the other diastereoisomer in DNA under the conditions of irradiation we employed. This complements a previous founding by two of us, L.V. and J.C., who did not detect stereoselective dTG formation in the gamma-irradiated dinucleoside monophosphate (TpT).[10] The radiogenic formation of the TG moiety in aerated solutions of DNA is thought to result from ·OH attack predominantly on the 5 position followed by O_2 fixation and subsequent hydroperoxide formation at the 6 position, the *trans* form predominating.[11] This then decomposes to the *trans* glycol with retention of configuration followed by epimerization to the *cis* form.[2] Thus, our results indicate that this *trans* attack is not stereoselective.

The dose-response curve we obtained is similar to that of Teoule *et al.*[12] who demonstrated a marked increase in TG formation in DNA at high doses of radiation. This was attributed to an increase in the single-stranded nature of the DNA with increasing radiation. This is particularly manifest in our experiment in the difference in percent (+, −) dTG formed in ss and ds DNA at 70 Gy. The G value we obtained for the formation of each isomer is 0.002, similar to that of another radiation-induced thymine derivative 5-hydroxymethyl-2′-deoxyuridine (HMdU).[9] However, it must be emphasized that the G value for dTG formation is probably favoured in ss DNA. Therefore, in cells where the DNA is not only ds but supercoiled, the formation of the TG moiety may be less efficient.

In contrast to radiation, the oxidation of thymidine by $KMnO_4$ is apparently stereoselective favoring the formation of the (−) *cis* dTG over the (+) *cis* dTG by 2:1. This indicates that there must be stereoselectivity in the formation of the cyclic MnO_4-thymidine intermediate formed during chemical oxidation. We have observed this phenomenon in chemically oxidized DNA with OsO_4 as well as $KMnO_4$.

We think that the relatively small amount formed of the tetra acetoxy derivatives

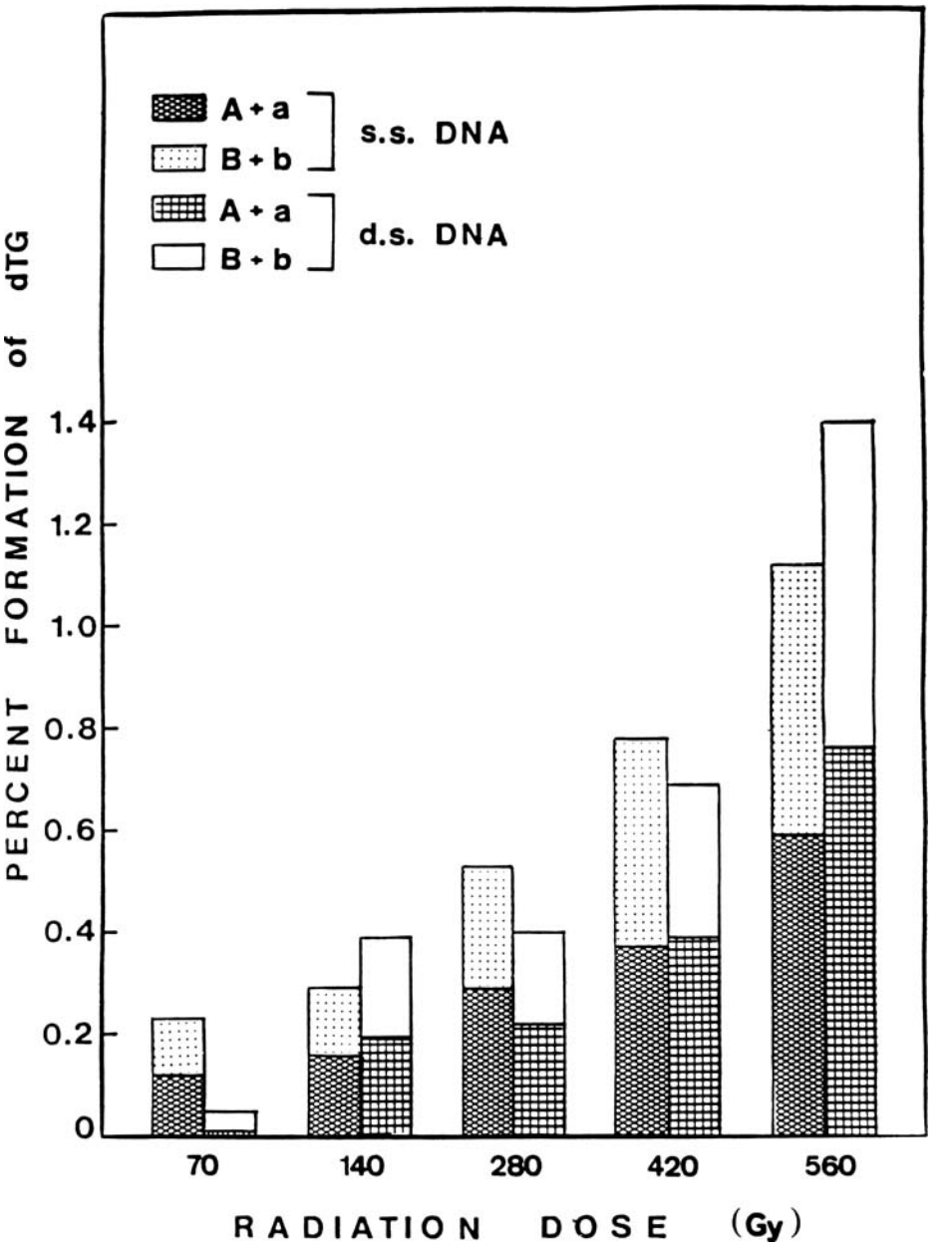

FIGURE 3 Dose response curve for the formation of (+) and (−) *cis* dTG in ss and ds gamma-irradiated [^{3}H] thymidine-labelled DNA. Irradiation was from a ^{137}Cs source at a rate of 4.5 Gy/min in aerated glass distilled H_2O. Determination of stereoisomer content made by the technique in Figure 1.

of (+) or (−) *cis* dTG is due to steric hindrance of the 5-OH by the methyl group of thymine. We could not effect an increased yield of this derivative by changing time or temperature nor by adding an even greater excess of acetic anhydride.

In summary, we have shown that the radiogenic formation of dTG in DNA is not stereoselective and that in ss DNA the G value for its formation is similar to HMdU. Both of these derivatives are subject to enzymatic repair through the action of N-glycosylases.[13–15] Using the technique of acetylation, we will be able to determine whether the enzymatic removal of the TG moiety in DNA is influenced by its configuration.

Acknowledgements

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