Synthesis of the diastereomers of thymidine glycol, determination of concentrations and rates of interconversion of their *cis-trans* epimers at equilibrium and demonstration of differential alkali lability within DNA

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ABSTRACT

5,6-dihydroxy-5,6-dihydrothymidine (thymidine glycol) is a major product of the reaction of thymidine with reactive oxygen species, including those generated by ionizing radiation. Thymidine glycol exists as 2 diastereomeric pairs by virtue of the chirality of the C(5) and C(6) atoms. A simple procedure is described for synthesizing and purifying each of the diastereomeric pairs separately. After brominating thymidine, the two *trans* 5-bromo-6-hydroxy-5,6-dihydrothymidine (thymidine bromohydrin) C(5) diastereomers were easily separated by High Performance Liquid Chromatography. Each thymidine bromohydrin was quantitatively converted to the corresponding diastereomeric thymidine glycol pair by reflux in aqueous solution. The concentrations at equilibrium of the *cis* (5S,6R), (5R,6S) and *trans* (5S,6S), (5R,6R) forms of the thymidine glycol diastereomers were determined and were 80% *cis* and 20% *trans* for the 5S pair and 87% *cis* and 13% *trans* for the 5R pair. At equilibrium, the rate of *cis-trans* epimerization of the two sets of diastereomers was essentially identical. The 5S diastereomeric pair was significantly more alkali labile than the 5R pair due to the higher concentration of the 5S *trans* epimer at equilibrium. This differential alkali lability was also manifest when the thymine glycol moiety was present in chemically oxidized poly(dA-dT)·poly(dA-dT) indicating that the chemical differences between the diastereomeric pairs are preserved in DNA. These chemical differences may affect the biological properties of this important oxidative derivative of thymine in DNA.

INTRODUCTION

5,6-Dihydroxy-5,6-dihydrothymine (thymine glycol) is formed in DNA as a product of oxidative free radical attack on the C(5)C(6) ethylenic bond of the thymine moiety (1,2). In DNA,

it is a major product of ionizing radiation (3,4,5) and other oxidative stress (6,7) and its biologic and mutagenic properties have been widely studied. The thymine glycol moiety in DNA blocks DNA polymerase action *in vitro* (8,9,10,11) and, when introduced into a unique site in a replicating vector, displays a low level of mutagenicity (12,13,14).

Thymine glycol is sufficiently chemically stable to be excised from DNA as the free modified base via the action of a DNA glycosylase activity found throughout nature (15–20) and has also been reported to be an *in vitro* substrate for the *uvr*(A)BC nucleotide excision repair complex of *E. coli* (21,22). Thymine glycol and thymidine glycol have been detected in the urine of laboratory animals fed a normal diet and in the urine of normal human subjects (23,24). Their presence has been attributed to their enzymatic removal from cellular DNA in which they were formed as a consequence of normal oxidative stress. Saul and Ames (24) have estimated that 400 thymine glycols are formed per cell per day. The recovery of thymine glycol and thymidine glycol from body fluids attests to the relative stability of the thymine glycol moiety. Nevertheless, when exposed to alkali, the thymine glycol ring fragments, leaving a urea residue composed of the N1-C2-N3 pyrimidine atoms bound to the 2'-deoxyribose (8,25).

In solution, thymidine glycol exists as two distinct diastereomeric pairs in equilibrium with each other: A (5S,6R;5S,6S) *cis-trans* pair and a (5R,6S;5R,6R) *cis-trans* pair. The most common laboratory synthesis of thymine glycol or thymidine glycol has been through the chemical oxidation of the thymine moiety using OsO₄ or KMnO₄ (4,26). This results in a mixture of both diastereomeric pairs which cannot be readily resolved by standard chromatographic techniques (5). Thus, studies which have introduced thymine glycol into DNA via chemical oxidation have measured the combined effects of both diastereomeric pairs.

We have developed a simple synthesis and purification of each of the diastereomeric pairs of thymidine glycol via bromination of thymidine (27,28,29) yielding the diastereomeric (5R,6R) and

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(5S,6S) *trans* bromohydrins. In contrast to the thymidine glycols, these can be readily separated by reversed phase High Performance Liquid Chromatography (HPLC). A simple aqueous reflux of each bromohydrin results in virtually quantitative yields of the corresponding *cis* and *trans* thymidine glycols. Thus, the chemical properties of each of the thymidine glycol diastereomers can be studied.

MATERIALS

[2-¹⁴C]Thymidine (58.3 mCi/mmol) was purchased from NEN Du Pont, Boston, MA. 'Suprapure' bromine was purchased from E. Merck, Darmstadt, Germany. Thymidine was purchased from Sigma, St. Louis, MO. Pyridine was purchased from J.T. Baker, Phillipsburg, NJ. Acetic anhydride was purchased from Aldrich, Milwaukee, WI.

METHODS

Synthesis of the [2-¹⁴C] *trans* (5R,6R) and *trans* (5S,6S) diastereomers of 5-bromo-6-hydroxy-5,6-dihydrothymidine (thymidine bromohydrin)

One g of thymidine was placed into a small pear shaped pyrex flask and 10 mL of distilled deionized water was added. Fifty μ Ci of [2-¹⁴C] thymidine was added (resulting in a specific activity of 12.1 μ Ci/mmol) and the mixture stirred with a magnetic stirrer for 20 min in an ice bath to reach 4°C. The suspension was then titrated dropwise with 'Suprapure' bromine using a long Pasteur pipette. As bromine was added, the relatively insoluble thymidine was converted to soluble thymidine bromohydrin. Bromine was added until the suspension cleared. A slight excess of bromine was added until a light red-brown color persisted. The brown solution was then stirred for a minimum of 3h in an ice bath, to insure that all the thymidine was brominated.

Purification, separation, and determination of the net yield of thymidine bromohydrins

After 3h of stirring, while still in the ice bath, nitrogen gas was bubbled through the brown solution for at least 1h to remove excess bromine. Removal was monitored by the disappearance of the red-brown color from the solution. At this time 1.2 mL of 5M sodium acetate was added to the now colorless bromohydrin solution bringing the pH to 4.0. To determine the yield, an aliquot of the reaction mixture was injected into an HPLC 4.6 mm $\times$ 25 cm ODS column using degassed 20% methanol/water as eluent at a flow rate of 1 mL/min and collecting 1 mL fractions. The radioactivity of each fraction was determined by liquid scintillation counting. To purify the two diastereomers, 500 μ L aliquots of the 10 mL reaction mixture were injected into a 10 mm $\times$ 25 cm ODS column using 20% methanol/water as eluent at a flow rate of 3 mL/min. Fractions corresponding to the UV absorbing peaks (226 nm) of the two isomers were collected manually and pooled. The identity of the two peaks was confirmed by analyzing each of the peaks by thin layer chromatography (TLC) (30).

Formation and purification of the [2-¹⁴C] *cis* (5S,6R) and *cis* (5R,6S) diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine (thymidine glycol)

Each pooled bromohydrin solution was dried under reduced pressure in a rotary evaporator and the volume quickly brought to 280 mL

with water. One molar ammonium bicarbonate was added until the pH reached 5.75. This solution was refluxed at a constant 100°C using a heating mantle. After 2h, the flask was removed from the mantle and cooled by standing at room temp. The final pH was 7.5. The volume was then reduced in a rotary evaporator to approximately 20 mL. To determine the yield, samples of each diastereomer were injected in a 4.6 mm $\times$ 25 cm ODS column using 100% water as eluent at a flow rate of 1 mL/min and 1 mL fractions were collected. Under these conditions, the *trans* (5R,6R) and *trans* (5S,6S) thymidine bromohydrin was converted to the *cis* (5S,6R) and *trans* (5S,6S) thymidine glycol. Similarly, a pair of *cis* (5R,6S) and *trans* (5R,6R) diastereomers was formed from the *trans* (5S,6S) thymidine bromohydrin. For either the R or S diastereomer two distinct UV (226nm) absorbing peaks were detected and all the radioactivity was present in these fractions. These two peaks represent the *cis* and *trans* epimers of thymidine glycol at carbon C(6). The two peaks were present for both diastereomers but their retention times varied slightly. As was done for the bromohydrins, the identity of each of the diastereomeric pairs was confirmed by thin layer chromatography (30).

Determination of the concentrations of the (5R,6S)–(5R,6R) and (5S,6R)–(5S,6S) pairs of *cis* and *trans* epimers at equilibrium

Three quantities of each diastereomeric pair ranging from 10³, 10⁴ and 10⁵ cpm were placed in 1 mL or 10mL (yielding concentrations ranging from 3.8 $\times$ 10^{–6} to 3.8 $\times$ 10^{–3}M) of pH 7.4, 0.01M phosphate buffer and allowed to incubate at 37°. Aliquots were sampled every 24 hours analyzed by HPLC, 0.5 mL fractions collected and the radioactive content of the *cis* and *trans* fractions determined by liquid scintillation counting. Determinations were made over a 72 hour period.

Determination of the rate of *trans* to *cis* epimerization for the (5R,6R)–(5R,6S) and (5S,6S)–(5S,6R) thymidine glycol diastereomers

A) *Purification of each of the trans epimers*: Approximately 10⁶ cpm of either diastereomer of HPLC purified *cis* thymidine glycol were collected in a 5mL pear shaped flask, and immediately dried on a rotary evaporator. The thymidine glycol was washed several times with 0.5 mL of freshly distilled dry pyridine and quickly redried on the evaporator to insure the absence of water. One mL of redistilled pyridine was then added to the flask and heated for 1h in a boiling water bath. The pyridine catalyzed epimerization reaction yielded about 30% *trans* form (31). The flask was removed and rotary evaporated to dryness. Two hundred fifty μ L of water was added and carefully swirled to redissolve the dried thymidine glycol. The entire volume was injected into a 10 mm $\times$ 25 cm ODS HPLC column using 100% water as eluent at a flow rate of 3 mL/min. The large peak corresponding to the *trans* isomer was collected. Approximately 2.5 $\times$ 10⁵ cpm of pure *trans* thymidine glycol were recovered for either diastereomer. A determined amount of phosphate buffer (pH 7.4) was added yielding a 0.01M solution which was 3.8 $\times$ 10^{–3}M in thymidine glycol.

B) *Determination of the initial rate of trans to cis epimerization*: Immediately after the phosphate buffer was added, repeated aliquots of solution containing 5 $\times$ 10³ cpm were analyzed in a 4.6 mm $\times$ 25 cm ODS HPLC column using 100% water at a flow rate of 1 mL/min, and collecting 1 mL fractions. By injecting 6 successive samples in 12 min intervals, the percent *trans* to

cis epimerization could be determined. This permitted calculation of the rate constant, k , for the epimerization of *trans* to *cis* thymidine glycol of each isomer. After plotting the decreasing $\log [\% \text{ trans}]$, a linear regression analysis was performed on the straight line formed. The rate constant, k , was determined from the slope of the straight line.

Formation and analysis of acetate derivatives of each diastereomer of thymidine glycol

Approximately 10^5 cpm of pure $[2-^{14}\text{C}]$ *cis* thymidine glycol was collected by HPLC. This was dried in a rotary evaporator and redissolved in 500 μL of dry, redistilled pyridine and 600 μL of redistilled acetic anhydride. This mixture was left overnight in the dark at 25°C . Five mL of water were added to decompose remaining acetic anhydride, and the volume reduced to 1 mL. Aliquots of 100 μL were analyzed by HPLC using a 30% methanol/70% water isocratic elution. This technique is identical to our previously published method (5) with the exception that methanol was substituted for acetonitrile.

Determination of differential alkali lability of the (5S,6R) and (5R,6S) diastereomers of *cis* thymidine glycol

Approximately 10^4 cpm of each diastereomer were collected as 100% *cis* by HPLC purification. The pH of these solutions was raised to 10 by a 0.01 M sodium carbonate/bicarbonate buffer and incubated at 37°C . To follow the disappearance of each *cis* thymidine glycol diastereomer, 2×10^3 cpm aliquots were periodically removed and injected into a 4.6 mm $\times$ 25 cm ODS HPLC column using 100% water as eluent at a flow rate of 1 mL/min and collecting 1 mL fractions. Radioactive content of the fractions was measured using a liquid scintillation counter.

Determination of differential alkali lability of the (5R,6S), (5S,6R) diastereomers of *cis* thymidine glycol in OsO_4 oxidized $\text{poly}(\text{dA}-[^3\text{H}]\text{dT}) \cdot \text{poly}(\text{dA}-\text{dT})$

Oxidized $\text{poly}(\text{dA}-[^3\text{H}]\text{dT}) \cdot \text{poly}(\text{dA}-\text{dT})$ was synthesized by the procedure described by (18). The only modification was the use of 10 M ammonium acetate in place of 3M sodium acetate to maximize the precipitation of $\text{poly}(\text{dA}-[^3\text{H}]\text{dT}) \cdot \text{poly}(\text{dA}-\text{dT})$. Aliquots of approximately 10^5 cpm of oxidized $\text{poly}(\text{dA}-[^3\text{H}]\text{dT}) \cdot \text{poly}(\text{dA}-\text{dT})$ were incubated at 37°C in a 50 mM sodium

carbonate/bicarbonate buffer pH 10.7. An aliquot was removed from the incubator after 0,1,2,3, and 4h. After removal, the solutions were brought to pH 7 with 1N HCl. The oxidized $\text{poly}(\text{dA}-[^3\text{H}]\text{dT}) \cdot \text{poly}(\text{dA}-\text{dT})$ from each aliquot was enzymatically digested to 2'-deoxyribonucleosides (4). After precipitation of the digestate with acetone, the supernatant was removed, evaporated to dryness and redissolved in 100 μL of water. One thousand cpm each of $[2-^{14}\text{C}]$ (5S,6R) and (5R,6S) *cis* thymidine glycol was added to each aliquot. The aliquots were then HPLC purified through a 4.6 mm $\times$ 25 cm ODS column with water as eluent. ^3H fractions which coeluted with ^{14}C marker were pooled and rotary evaporated to dryness. The amount of $[^3\text{H}]$ thymidine glycol remaining after alkaline incubation was determined by derivatizing the pooled HPLC purified fractions from each aliquot with acetic anhydride and pyridine as described above. The differential loss of each C(5) diastereomer with time was recorded by comparing the amount of $[^3\text{H}]$ recovered with that of the added $[^{14}\text{C}]$ thymidine glycol.

RESULTS

Synthesis and purification of the diastereomers of *trans* thymidine bromohydrin

Purification of the (5R,6R) and (5S,6S) *trans* thymidine bromohydrin diastereomers was accomplished by HPLC separation using absorbance at 226 nm to monitor separation. In a sample run of 50,000 cpm injected, 12,800 cpm were recovered under the (5S,6S) *trans* thymidine bromohydrin peak (retention time of 7.75 min) and 28,600 cpm under the (5R,6R) *trans* thymidine bromohydrin peak (retention time of 11.62 min) totalling 41,400 cpm for an 83% yield. The 2:1 ratio of 5R:5S isomer is characteristic of the yield of bromohydrins obtained by bromination of thymidine. The identity of each peak was confirmed by their migration on thin layer chromatograms using the system described by Cadet and Teoule (30). After purification, of the original 10^8 cpm, 4.8×10^7 cpm (48%) was recovered as (5R,6R) *trans* thymidine bromohydrin and 2.7×10^7 cpm (27%) as (5S,6S) *trans* thymidine bromohydrin.

Synthesis and purification of the diastereomers of thymidine glycol

After HPLC separation of each bromohydrin and neutralization of the HBr produced within the bromohydrin reaction, each was quantitatively converted to the corresponding thymidine glycol by boiling in water under a reflux condenser. The thymidine glycol epimers were isolated from the reaction mixture using absorbance at 226 nm to monitor separation. The total yield of each thymidine glycol diastereomeric pair from the corresponding bromohydrin was approximately 65–70%. The retention time of the *trans* (5S,6S) isomer was 5.25 min while that of the *cis* (5S,6R) was 11.51 min. For the *trans* (5R,6R) the retention time was 6.16 min and 11.92 min for *cis* (5R,6S) thymidine glycol. Since differences in retention time of less than 0.5 min preclude satisfactory resolution of peaks, only the respective *trans* peaks are distinguishable from each other in a mixture of the two diastereomeric pairs.

Derivatization of thymidine glycol via acetylation

However, it is possible to quantitatively distinguish one diastereomeric pair from the other using derivatization. Acetylation yields derivatives of each diastereomer which are readily separable (5). Such a separation is shown in Figure 1.

Figure 1. HPLC separated radioactivity profile of the acetate derivatives of the (5S,6R) thymidine glycol (open circles) and (5R,6S) thymidine glycol (closed circles). One mL samples were collected from the HPLC and after addition of scintillant were counted in a liquid scintillation counter.

Figure 2. Plot of $\log [\% \text{ trans}]$ vs time for the (5R,6R) and (5S,6S) thymidine glycols. (Closed circles) $\log [\% (5S,6S)]$; slope = 3.50×10^{-3} ; (Open circles) $\log [\% (5R,6R)]$; slope = 5.84×10^{-3} .

This illustrates the radioactivity profile of the acetate derivatives of the (5S,6R) and (5R,6S) *cis* thymidine glycols. The large peaks represent the 3',5' diacetates and the smaller peaks the 3',5',6 triacetates and the 3',5',5 and 6 tetraacetates(36). The retention times of these peaks were very similar to those we previously reported for the acetylation products of a mixture of (5R,6S) and (5S,6R) *cis* thymidine glycols (5).

Determination of the concentrations at equilibrium of each of the *cis* and *trans* thymidine glycol diastereomeric pairs

Determination of the concentrations of the respective *cis* and *trans* epimers was accomplished by sampling the pH 7.4 solution over a period of several days and analyzing the radioactive content of the eluent fractions separated by HPLC. Within 24 hours after HPLC purification of the *cis* thymidine glycol from the original bromohydrin reaction mixture, the relative concentrations of the (5S,6R) and (5S,6S) pair were 80% *cis* and 20% *trans* and for the (5R,6S) and (5R,6R) pair 87% *cis* and 13% *trans*. These values remained constant over the next 48 hours and were constant over 4 orders of magnitude of concentration.

Determination of the rate of epimerization for each of the *cis-trans* diastereomeric pairs

To determine the initial rate of *trans* to *cis* epimerization, the *trans* epimer of each diastereomeric pair was isolated and the rate of appearance of the corresponding *cis* epimer measured by HPLC analysis. Since the concentrations of the *trans* forms at equilibria are very low when compared to those of the *cis* forms, the rate of epimerization from *trans* to *cis* could be much more accurately measured than the reverse *cis* to *trans* reaction. Six determinations were done within the first 90 min and these points used to determine k . At this early time the reverse epimerization reaction of *cis* to *trans* is negligible. The plot of $\log \%$ (5R,6R) and (5S,6S) *trans* thymidine glycols vs time is shown in Figure 2. The linear regression reveals a k of $3.50 \times 10^{-3} \text{ min}^{-1}$ for the (5S,6S) diastereomer and a k of $5.84 \times 10^{-3} \text{ min}^{-1}$ for the (5R,6R) diastereomer.

The rate of epimerization of each isomer at equilibrium was determined from the equation: Rate = $k[\% \text{ trans}]$. By inserting

Figure 3A. Semi-log plot of percent radioactivity vs time, of the disappearance of the (5S,6R), (5S,6S) and (5R,6S), (5R,6R) thymidine glycol diastereomeric pair. (Closed circles) % (5S,6R), (5S,6S); (Open circles) % (5R,6S), (5R,6R). **B.** Semi-log plot of radioactivity vs time, of the disappearance of the (5S,6R) and (5R,6S) thymidine glycols from poly(dA-[³H]dT)·poly(dA-dT). (Closed circles) (5S,6R) thymidine glycol; (open circles) (5R,6S) thymidine glycol. The relative percentage of each *cis* diastereomer left in the polynucleotide with time was determined by enzymatic digestion, HPLC purification of the 5R and 5S thymidine glycol diastereomers followed by acetylation and HPLC analysis similar to that demonstrated in Figure 1. Recovery was monitored by putting in a known amount of 5R and 5S ¹⁴C diastereomers prior to acetylation and measuring the ratio of ³H-containing polynucleotide derived thymidine glycol acetate to ¹⁴C authentic thymidine glycol acetate.

the values for k and for percent *trans* at equilibrium, the rate of *trans* to *cis* epimerization at equilibrium was determined to be 0.076%/min for 5R thymidine glycol and 0.069%/min for 5S thymidine glycol.

Determination of the relative alkali lability of the 5S and 5R thymidine glycol *cis-trans* diastereomeric pairs

To measure alkali lability, each diastereomeric pair was incubated as described in 'Methods' and samples analyzed by HPLC. For

Figure 4. Mechanism of epimerization between the *cis* and *trans* forms of the 5R and 5S thymidine glycol diastereomers.

each point, all the radioactivity was recovered in either the *cis* and *trans* thymidine glycol peaks. It is known that alkaline hydrolysis of thymine glycol yields urea composed of the N1-C2-N3 atoms of the pyrimidine ring (8,25). Similarly, when thymidine glycol is exposed to alkali, 2'-deoxyribosylurea is formed. This compound cannot be separated from the *trans* nucleoside by reversed phase HPLC and the two elute together.

Since the urea nucleoside breakdown product of thymidine glycol co-elutes with the respective *trans* peak, on reversed phase HPLC, le Chatelier's Principle was applied to the determination of the actual amount of thymidine glycol breakdown. Since the amount of each *cis* thymidine glycol epimer was readily measured on the HPLC, the amount of *trans* could be calculated from the previously determined equilibrium concentrations. Thus, the total amount of residual *cis* and *trans* thymidine glycol could be determined for each time point.

The rate of disappearance of the total amount of *cis* and *trans* thymidine glycol for both diastereomeric pairs is illustrated in Figure 3A. It is evident that there is differential alkali lability between the (5R,6S), (5R,6R) and (5S,6R), (5S,6S) thymidine glycol diastereomeric pairs measured at pH 10, the 5S diastereomeric pair being much more alkali labile than the 5R pair.

Determination of the relative alkali lability of the 5S and 5R thymidine glycol *cis-trans* diastereomeric pairs within DNA

To determine whether this differential alkali lability was manifest when the thymine glycol moiety was on the DNA backbone, OsO₄ oxidized poly(dA-[³H]dT)·poly(dA-dT) heteroduplexes were made as described by Higgins *et al* (18). Chemical oxidation by OsO₄ introduces both 5S and 5R diastereomers into DNA. The oxidized double stranded polynucleotide was incubated at

pH 10 which yielded relatively little breakdown of thymine glycol. However, raising the pH to 10.7 was sufficient to effect alkaline decomposition of the thymine glycol moiety as seen in Figure 3B. The lines represent the decrease in recoverable ³H radioactivity under a peak of the acetate derivative of each diastereomer of thymidine glycol coeluting with the acetate derivative of the [¹⁴C] *cis* thymidine glycol. The rate of loss of [³H] *cis* thymidine glycol was calculated from the ratio of ³H dpm/¹⁴C dpm of each acetate peak. As was the case for the free nucleosides in solution, the (5S,6R) diastereomer was more alkali labile than the (5R,6S) diastereomer.

DISCUSSION

The thymine glycol moiety is the major product of oxidative attack on thymine and is a substrate for both prokaryotic and eukaryotic DNA repair enzymes (Reviewed in (32)). The finding of only one thymidine glycol diastereomer in human urine (24) suggested that formation and/or repair of thymidine glycol in cellular DNA might be stereoselective. Using acetylation as an analytical technique, we previously demonstrated that the (5R,6S) and (5S,6R) *cis* thymidine glycols were formed in equal amounts in DNA exposed to ionizing radiation *in vitro* (5). Since the mechanism of formation of thymidine glycol *via* the indirect action of ionizing radiation is similar to its formation by endogenous oxidative stress, it seemed unlikely that the observed differential excretion was a reflection of differential formation. For this reason we undertook a study of the relative stability of the two diastereomeric pairs.

Because formation of thymidine glycol via oxidation by KMnO₄ or OsO₄ yields a mixture of both 5R and 5S diastereomeric pairs which are very difficult to separate by HPLC we turned to a

synthesis which yields each of the diastereomeric pairs of thymidine glycol separately via bromination of thymidine (30).

We modified the original synthesis in two ways. The first was the use of HPLC in place of TLC to separate the *trans* bromohydrins. This greatly increased the capacity for synthesizing large amounts of the compounds. The second modification was the use of a buffered aqueous reflux instead of an aqueous pyridine reflux. This reduced the yield of by products thereby increasing the yield of thymidine glycol.

The generation of thymidine glycol from brominated thymidine is stereospecific at C(5) resulting in inversion of configuration (5R bromohydrin to 5S glycol and *vice versa*) due to an S_N2 attack of the C(6)OH of the bromohydrin on the C(5)Br atom (29,30,33). In the synthesis described here, once formed, each of the *cis* and *trans* thymidine glycol epimers of the 5R and 5S diastereomeric pairs immediately reach equilibrium due to the mildly alkaline pH which develops during boiling.

The mechanism of the specific isomerisation in aqueous solution of each of the relatively unstable *trans* thymidine glycol diastereomers into the correspondingly more stable *cis* forms was elucidated through the use of ^{18}O (29). About 50% of the label from the C(6) position was lost upon heating of the *cis* thymine glycol to form the *trans* epimer. This led to postulation of the mechanism shown in Figure 4. Epimerization at C(6) probably takes place through water addition to the C(6)-N(1) bond forming a geminal diol on C(6) and proton addition to N(1).

We suggest that it is the degree of epimerization at C(6) which accounts for the greater rate of degradation to the 2'-deoxyribosylurea of the 5S thymidine glycol pair as compared to the 5R thymidine glycol pair. Since the equilibrium concentration of the (5S,6S) *trans* glycol is almost twice that of the (5R,6R) *trans* glycol, twice as much of the 5S compound is in the transient ring open form shown in Figure 4 at any one time, favoring fragmentation to 2'-deoxyribosylurea under alkaline conditions.

In poly (dA-dT)·poly(dA-dT) the thymine glycol moiety was not degraded until the pH was raised to 10.7 as compared to 10 for the nucleoside. This suggests that hydrogen bonding and base stacking interactions contribute to the reduced alkali lability of the thymine glycol moiety in DNA as compared to thymidine glycol in solution. Although there is no data yet available, one must consider the possibility that, in native DNA, sequence may also influence the alkali lability of the thymine glycol moiety.

What is the relevance of this data to biologic systems? Previously, both Cadet and Voituriez (34) and Sharma *et al* (35), using NMR, detected *cis* and *trans* thymidine glycol within thymidine containing oligonucleotides which had been exposed to ionizing radiation while in solution. We interpret the finding of differential alkali lability of the thymine glycol moiety within oxidized poly (dA-dT)·poly(dA-dT) as evidence that the thymidine glycol diastereomers within DNA are in the same *cis-trans* equilibrium as are the diastereomeric thymidine glycol nucleosides in solution. Thus, models of thymidine glycol within DNA which depict the *cis* or *trans* diastereomers in static conformational states are not representative of the dynamic epimerization equilibrium state.

Secondly, the synthesis of the individual thymidine glycol diastereomers via the bromohydrin methodology and the determination of their equilibrium concentrations improves the quantitation of thymidine glycol in DNA through acetylation (5). Frenkel *et al* (36) have devised a derivatization scheme using 3H -acetic anhydride to assess the formation of glycols and other

oxidized bases in non-radioactively labeled DNA. Small amounts of each of the ^{14}C labeled diastereomers could be added to such a mixture as controls for the efficiency of the acetylation reaction. In addition, since the equilibrium concentrations of each *cis-trans* diastereomeric pair are now known, the total amount of thymidine glycol formed under the experimental conditions employed can be calculated with greater accuracy.

This strategy may also be applied to determine whether enzymatic release of thymine glycol is influenced by its conformation within DNA. The modified base, thymine glycol, is released from DNA via base excision repair by a transiminization mechanism through the action of endonuclease III of *E.coli* (37). Because the thymine glycols released from the DNA backbone are enantiomers (18), they cannot be separated by non-chiral columns. However, one could examine the relative content of the diastereomers of thymidine glycol within the oxidized poly (dA-dT)·poly(dA- 3H]dT) substrate before and after incubation with endonuclease III. A significant change in the ratio of the 5R to 5S diastereomers would suggest that endonuclease III mediated repair was stereoselective.

Recently, it was reported that thymidine glycol is also a substrate for the *E.coli* *uvr*(A)BC complex which excises 'bulky' adducts such as pyrimidine dimers from DNA via nucleotide excision repair (21,22). Cathcart *et al* (23) reported the identification of both thymine glycol and thymidine glycol in the urine of normal laboratory animals and human volunteers. The presence of thymine glycol could be attributed to base excision by an endonuclease III type activity known to be present in mammalian cells (18,19,20). The thymidine glycol might result from excision via the nucleotide excision pathway. Its appearance in the urine might be due to nucleolytic and phosphatase mediated degradation of the oligonucleotide containing the thymidine glycol which had originally been excised from the DNA backbone by the nucleotide excision excinuclease. Perhaps the finding of one of the two diastereomers of thymidine glycol in the urine in great excess over the other (24) results from differential excision repair. It is possible that conformational and/or chemical differences between the 5R and 5S diastereomeric pairs may influence their relative rate of repair by the two excision repair pathways. This possibility is currently being explored using the techniques described in this report.

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