# Synthesis of Dihydrothymidine and Thymidine Glycol 5′-Triphosphates and Their Ability To Serve as Substrates for *Escherichia coli* DNA Polymerase I†

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ABSTRACT: 5,6-Dihydrothymidine 5′-triphosphate (DHdTTP) was synthesized by catalytic hydrogenation of thymidine 5′-triphosphate (dTTP). Thymidine glycol 5′-triphosphate (dTTP-GLY) was prepared by bromination of dTTP followed by treatment with $Ag_2O$. The modified nucleotides were extensively purified by anion-exchange high-performance liquid chromatography (HPLC). Alkaline phosphatase digestion of DHdTTP and dTTP-GLY gave the expected products (5,6-dihydrothymidine and *cis*-thymidine glycol), the identities of which were confirmed by reverse-phase HPLC using authentic markers. HPLC analysis of the alkaline phosphatase digested DHdTTP revealed that DHdTTP was a mixture of C5 diastereoisomers [(5*S*)- and (5*R*)-DHdTTP]. Despite the significant distortion of the pyrimidine ring in DHdTTP, it was incorporated in place of dTTP during primer elongation catalyzed by *Escherichia coli* DNA polymerase I Klenow fragment. The rate of incorporation of DHdTTP was about 10–25-fold lower than that of dTTP. On the other hand, dTTP-GLY, which also has a distorted pyrimidine ring, did not replace dTTP, and no elongation of the primer was observed. In order to study the preference of incorporation of the diastereoisomers of DHdTTP into DNA, salmon testes DNA, activated by exonuclease III, was used as a template for DNA polymerase I Klenow fragment in the presence of [$^3$H]DHdTTP (*S* and *R* mixture) and normal nucleotides. After enzymatic digestion of the DNA to nucleosides, the products were analyzed by HPLC. The ratio of the isomers incorporated into DNA (*S*:*R* = 73:27) was virtually the same as that of the [$^3$H]DHdTTP substrates (*S*:*R* = 79:21). This result suggests that *Escherichia coli* DNA polymerase I uses both isomers of DHdTTP as substrates and that the overall efficiency of incorporation is primarily determined by the concentration of the isomers in the nucleotide pool.

Ionizing radiation produces different classes of DNA damages such as base damages, apurinic/apyrimidinic (AP)[1] sites, and single- and double-strand breaks (Ward, 1975, 1981). These lesions are believed to lead to mutagenic or lethal events in cells by changing the coding properties of DNA bases or constituting replicative blocks. One of the difficulties in assessing the biological consequences of individual radiolysis products in DNA is that very few methods are available to produce unique damages. Thus far *cis*-thymine glycol (Hariharan et al., 1977; Moran & Wallace, 1985; Ide et al., 1985; Rouet & Essigman, 1985; Hayes & LeClerc, 1986; Clark & Beardsley, 1986), urea (Ide et al., 1985; Hayes & LeClerc, 1986; M. F. Laspia, L. Petrullo, and S. S. Wallace, unpublished results), and AP sites (Schaaper & Loeb, 1981; Moran & Wallace, 1985; Strauss et al., 1982; Sagher & Strauss, 1983) have been produced in phage DNA, and their biological consequences have been assessed by use of in vitro DNA replication systems or phage transfection systems. Although the primary product produced in DNA by $OsO_4$ oxidation is *cis*-thymine glycol (Beer et al., 1966; Frenkel et al., 1981), even in this case the mutagenic lesion appears to be a minor cytosine modification (R. C. Hayes, H. Huang, S. S. Wallace, and J. E. LeClerc, unpublished results). Thus alternative approaches are necessary.

In this paper we describe the synthesis and extensive high-performance liquid chromatography (HPLC) purification of the 5′-triphosphates of 5,6-dihyrothymidine (DHdTTP) and thymidine glycol (5,6-dihydroxy-5,6-dihydrothymidine, dTTP-GLY). These compounds were synthesized in order to measure their ability to serve as substrates for DNA polymerase in vitro so that they might be specifically engineered into DNA. Both 5,6-dihydrothymine and thymine glycol have a saturated C5–C6 double bond and are stable. The former is a major radiolysis product produced in DNA under anoxic conditions (Têoule et al., 1978; Dizdaroglu, 1985; Furlong et al., 1986), while the latter is produced under oxic conditions (Têoule et al., 1977).

## Materials and Methods

*Chemicals.* The HPLC-purified 2′-deoxynucleoside 5′-triphosphates (dNTP) were obtained from P-L Biochemicals, and thymine, 5,6-dihydrothymine, and thymidine were from Sigma. [*methyl*-$^3$H]dTTP (62 Ci/mmol) and [5-$^3$H]dCTP (28 Ci/mmol) were purchased from ICN. Rhodium catalyst (5% rhodium on aluminum) was obtained from Aldrich, and DEAE-Sephadex A-25 was a product of Pharmacia. PEI–cellulose TLC plates were purchased from Macherey-Nagel. A mixture of (5*S*)-(−)- and (5*R*)-(+)-5,6 dihydrothymidine was prepared by hydrogenation of thymidine with rhodium catalyst (Cohn & Doherty, 1956). In the reverse-phase HPLC analysis of the diastereoisomers of dihydrothymidine, the retention time of *S*-(−) isomer (13.5 min), which is known to be preferentially formed in the hydrogenation with rhodium

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[1] Abbreviations: HPLC, high-performance liquid chromatography; dNTP, 2′-deoxynucleoside 5′-triphosphate; dTTP, thymidine 5′-triphosphate; DHdTTP, 5,6-dihydrothymidine 5′-triphosphate; dTTP-GLY, thymidine glycol 5′-triphosphate; dATP, 2′-deoxyadenosine 5′-triphosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; pDAB, *p*-(dimethylamino)benzaldehyde; Tris, tris(hydroxymethyl)aminomethane; AP, apurinic/apyrimidinic; DEAE, diethylaminoethyl; PEI, poly(ethylenimine); TLC, thin-layer chromatography; TEAB, triethylammonium bicarbonate.

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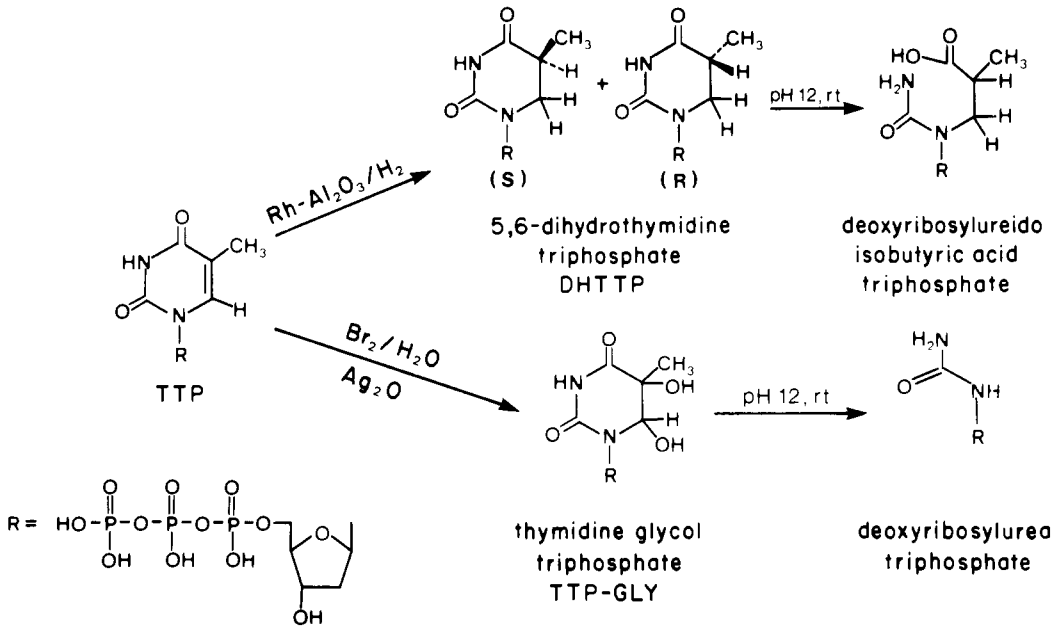


FIGURE 1: Synthesis and alkali hydrolysis of 5,6-dihydrothymidine (DHdTTP) and thymidine glycol (dTTP-GLY) 5′-triphosphates.

catalyst (Kondo & Witkop, 1968; Konnert et al., 1970), was shorter than that of *R*-(+) isomer (15.7 min; for experimental detals see below). Thus, the assignment of the elution peaks of diastereoisomers in the reverse-phase HPLC analysis reported by Cadet et al. (1981) should be reversed. The present assignment of the isomers is in accord with the report by Nishimoto et al. (1983a). *cis*-Thymine glycol and *cis*-thymidine glycol were synthesized by permanganate oxidation of thymine and thymidine, respectively, and further purified by cellulose column chromatography (Iida & Hayatsu, 1970, 1971). All other reagents were of the best available grades.

*Enzymes and DNA.* Alkaline phosphatase, micrococcal nuclease, exonuclease III, and *Escherichia coli* DNA polymerase I Klenow fragment were obtained from Pharmacia. Salmon testes DNA and spleen phosphodiesterase were obtained from P-L Biochemicals. For the polymerase reaction, salmon testes DNA was activated by extensive exonuclease III digestion. M13mp11 DNA template, primed with synthetic 17-mer [New England BioLabs M13 sequencing primer (−20)], was prepared as previously described (Ide et al., 1985).

*Preparation of DHdTTP and dTTP-GLY.* DHdTTP was prepared by following the method of Cohn and Doherty (1956) (Figure 1). An aqueous solution (20 mL) containing dTTP (50 mg) and rhodium catalyst (40 mg) in a 1-L flask was purged with hydrogen gas for 30 min and tightly capped. After the solution was stirred for 24 h, the catalyst was removed by centrifugation. The complete conversion of dTTP was confirmed by the loss of UV absorption maximum at 267 nm. The solution was evaporated at room temperature, and the resulting viscous material was dissolved in 5 mL of water. The solution was filtered with a 0.45-$\mu$m filter and subjected to HPLC purification. [$^3$H]DHdTTP was synthesized by catalytic hydrogenation of [$^3$H]dTTP (100 $\mu$Ci) by using a method similar to that used for the unlabeled compound. After the catalyst was removed by centrifugation, the reaction solution was loaded onto a DEAE-Sephadex A-25 column (1 × 4 cm) equilibrated with triethylammonium bicarbonate (TEAB; pH 7.5). The elution was carried out with a linear gradient of TEAB (0.02–0.6 M, 200 mL). The fractions containing [$^3$H]DHdTTP (0.35–0.45 M) were combined and evaporated at room temperature. The buffer component was removed completely by repeated evaporation with methanol. Finally, [$^3$H]DHdTTP was dissolved in a small quantity of water and stored at −20 °C. Further purification with HPLC was not performed.

The method for the preparation of dTTP-GLY was essentially the same as described for thymidine glycol 5′-monophosphate (Rajagopalan et al., 1984) (Figure 1). dTTP (50 mg) was dissolved in water (1 mL), and bromine (30 $\mu$L) was added to the solution. The solution was well mixed and kept at room temperature for 45 min. The unreacted bromine was extracted by chloroform, and the aqueous solution was purged with nitrogen gas to remove trace bromine and chloroform. Silver oxide ($Ag_2O$), prepared from silver nitrate (50 mg) and concentrated aqueous NaOH, was added to the aqueous solution, and the mixture was kept in the dark overnight at room temperature with stirring. $Ag_2O$ was removed by centrifugation, and the reaction solution was purged with hydrogen sulfide ($H_2S$) gas. The resulting dark precipitate of silver sulfide was removed by centrifugation. The solution was filtered with a 0.45-$\mu$m filter and subjected to HPLC purification.

*HPLC Purification.* DHdTTP and dTTP-GLY were purified by high-performance liquid chromatography (LDC/Milton Roy) equipped with an anion-exchange column (SOTA AX 300; 10 × 250 mm). The elution peaks were monitored by UV absorption at 210 or 260 nm with an LDC/Milton Roy variable-wavelength detector. Peak areas were integrated with the CCM HPLC automation system. In each HPLC run about 4 mg of the modified nucleotide was loaded onto the column. The elutions were carried out isocratically at a flow rate of 7 mL/min with 50 mM $NaH_2PO_4$ (pH 5.9), 150 mM $(NH_4)_2SO_4$, and 30 vol % methanol. The pooled fractions containing DHdTTP or dTTP-GLY were combined, diluted 5-fold with water, and loaded onto a DEAE-Sephadex A-25 column (2.5 × 9 cm) equilibrated with TEAB. The column was washed first with 50 mM TEAB (500 mL) and then with 1 M TEAB (200 mL). The 1 M fractions (25 mL) were collected. The fractions containing DHdTTP and dTTP-GLY were detected by diphenylamine reagent (Schneider, 1945). The combined fractions (the third and fourth fractions) of DHdTTP or dTTP-GLY were evaporated at room temperature. After the addition of methanol, evaporation was repeated until the buffer component was removed completely. dTTP-

GLY was then subjected to a second HPLC purification. The procedures for the second runs were the same as those described for the first runs. Finally, DHdTTP and dTTP-GLY were converted to their sodium salts (Hoard & Ott, 1965).

*Alkaline Phosphatase Digestion of DHdTTP and dTTP-GLY.* In order to confirm the structure of DHdTTP and dTTP-GLY, DHdTTP and dTTP-GLY (about 200 μg) were digested with alkaline phosphatase (4 units). Incubation was carried out in Tris·HCl (50 mM, pH 8.0) at 37 °C for 1 h. The reaction solutions were passed through a mini DEAE-Sephadex A-25 column ($HCO_3^-$ form; 1 mL) and evaporated. The digested products were dissolved in aliquots of water and analyzed by HPLC using a Whatman reverse-phase Partisil 5 ODS-3 (4.6 × 250 mm) column. The elution was carried out with water–methanol (95:5) at a flow rate of 0.8 mL/min. The elution peaks were monitored by UV absorption at 210 nm.

*Alkali Hydrolysis of DHdTTP and dTTP-GLY.* HPLC-purified DHdTTP and dTTP-GLY (about 50 μg) were dissolved in 0.2 mL of phosphate buffer (0.2 M, pH 12.0) and incubated at room temperature for 2 h. An aliquot of the reaction solution was spotted onto a silica gel TLC plate. After the plate was air-dried, an ethanol solution of *p*-(dimethylamino)benzaldehyde (pDAB), which consists of 1 g of pDAB and 10 mL of concentrated HCl per 100 mL, was sprayed on the spot.

*Measurement of the Ability of DHdTTP and dTTP-GLY To Serve as Substrates for DNA Polymerase.* The ability of DHdTTP and dTTP-GLY to serve as substrates for DNA polymerase was measured by their rate of incorporation into DNA catalyzed by *E. coli* DNA polymerase I Klenow fragment. The reaction mix (10 μL) contained M13mp11 DNA templates (0.5 μg) primed with synthetic 17-mer, 10 μM of three dNTPs [dATP, dGTP, dCTP ($3 \times 10^4$ cpm/pmol)], dTTP or a modified nucleotide (0–100 μM), DNA polymerase I Klenow fragment (0.5 unit), Tris·HCl (50 mM, pH 8.0), and $MgCl_2$ (8 mM). Incubation was carried out at 25 °C. At appropriate reaction times, an aliquot (2 μL) of the reaction solution was removed and spotted on a Whatman GF/A filter. The filters were washed by trichloroacetic acid and then by ethanol and assayed for radioactivity.

*Preparation and Enzymatic Digestion of DNA Containing [$^3H$]Dihydrothymidine.* Salmon testes DNA (15 μg), activated by exonuclease III treatment, was replicated by DNA polymerase I Klenow fragment (22 units) in the presence of three dNTPs [dATP, dGTP, dCTP (20 μM)], dTTP (0.1 μM), DHdTTP ($10^6$ cpm), Tris·HCl (50 mM, pH 8.0), and $MgCl_2$ (8 mM). Incubation was carried out at 25 °C for 1 h. The DNA was ethanol-precipitated, and the DNA pellet was washed by 70% ethanol. The DNA was dissolved in water and stored at −20 °C. The DNA containing [$^3$H]dihydrothymine (5 μg, $10^5$ cpm) in 40 μL of $CaCl_2$ (3.3 mM) and Tris·HCl (25 mM, pH 7.5) was digested to nucleoside 3′-monophosphates by using micrococcal nuclease (100 units) and spleen phosphodiesterase (0.01 unit) as described by Franklin and Haseltine (1983). Subsequently, alkaline phosphatase (0.4 unit) was added to the reaction mix and incubation was continued further for 1 h at 37 °C. In order to confirm the complete conversion of DNA to nucleosides, an aliquot (2 μL) of the reaction solution was analyzed by PEI–cellulose TLC with methanol as a developing solvent. In this analysis, DNA, oligonucleotides, and nucleoside monophosphates are retained at the origin while nucleosides are eluted from the origin. We found that less than 5% of the total radioactivity was retained at the origin after methanol elution, indicating that the digestion was over 95% complete. As a control, [$^3$H]DHdTTP was also digested with alkaline phosphatase as described for unlabeled DHdTTP. After digestion of $^3$H-labeled DNA and DHdTTP, the reaction solutions were passed through a DEAE-Sephadex A-25 column ($HCO_3^-$ form; 0.1 mL) to remove enzymes, and the column was washed by water (100 μL). The eluents were mixed with markers and analyzed by reverse-phase HPLC (see Alkaline Phosphatase Digestion of DHdTTP and dTTP-GLY). Four drops of the column eluents were collected, and the radioactivity was measured.

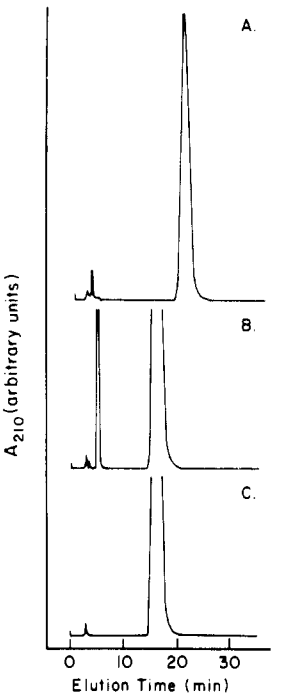


FIGURE 2: HPLC analysis of dTTP (A) and DHdTTP before (B) and after (C) HPLC purification. In (C), HPLC-purified DHdTTP was rechromatographed under the same elution conditions described in Materials and Methods. Under these conditions, dTTP and DHdTTP were eluted with retention times of 21.0 and 15.7 min, respectively. All elution profiles were normalized with respect to the peak intensities by using the replotting program associated with the LDC/Milton Roy chromatograph control module.

## RESULTS

*HPLC Purification of DHdTTP and dTTP-GLY.* Since the contamination of dTTP in DHdTTP and dTTP-GLY could lead to an invalid conclusion about the ability of the modified nucleotides to serve as substrates for DNA polymerases, DHdTTP and dTTP-GLY were extensively purified by HPLC. Figure 2 shows the typical HPLC elution profiles of dTTP (A) and DHdTTP before (B) and after (C) HPLC purification. Since DHdTTP has no UV absorption maximum at 267 nm due to the saturation of the C5–C6 double bond, elution products were monitored by the UV absorption at 210 nm. From Figure 2A,B it is clear that catalytic hydrogenation of dTTP yielded DHdTTP (retention time 15.7 min) as a single major product. The minor reaction product was eluted at a considerably shorter retention time (4.9 min). When elution peaks were monitored at 260 nm, the product showed an extremely weak detector response compared with that at 210 nm. This result suggests saturation of the C5–C6 double bond in the product. Partial breakdown of the triphosphate group was also indicated by its short retention time relative to dTTP and DHdTTP. Thus it is likely that the minor product is 5,6-dihydrothymidine 5′-mono- or 5′-diphosphate. Before

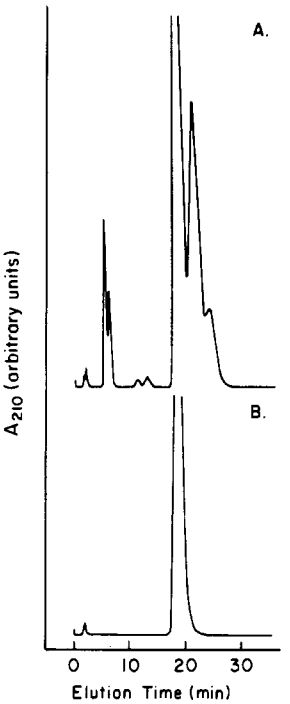


FIGURE 3: HPLC analysis of dTTP-GLY before (A) and after (B) two cycles of HPLC purification. In (B), HPLC-purified dTTP-GLY was rechromatographed under the same elution conditions described in Materials and Methods. Under these conditions, dTTP-GLY was eluted with a retention time of 18.2 min. Elution profiles were normalized with respect to the peak intensities by using the replotting program with the LDC/Milton Roy chromatograph control module.

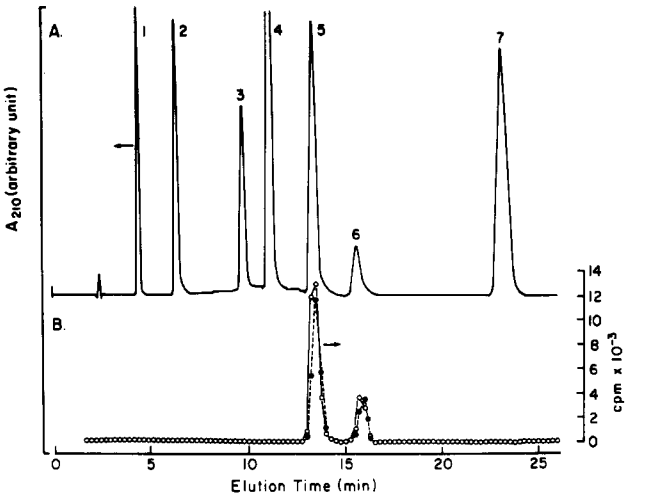


FIGURE 4: HPLC analysis of digested DHdTTP and DNA containing [$^3$H]dihydrothymidine. Panel A shows the elution profile of markers detected by UV absorption at 210 nm: 1, *cis*-thymine glycol; 2, *cis*-thymidine glycol; 3, 5,6-dihydrothymine; 4, thymine; 5, (5*S*)-(−)-5,6-dihydrothymidine; 6, (5*R*)-(+)-5,6-dihydrothymidine; 7, thymidine. Panel B shows the radioactivity from each fraction for digested DHdTTP (○) and DNA (●). The digested samples were mixed with markers 1–7 and injected into HPLC.

HPLC purification, the contamination of dTTP (retention time 21.0 min) in DHdTTP was estimated to be about $10^{-3}$, on the basis of the integrated areas of DHdTTP and presumptive dTTP peaks. We found that it was important to reduce dTTP completely to increase the efficacy of the subsequent HPLC purification. If the reduction of dTTP was incomplete, a second cycle of HPLC purification was necessary to achieve satisfactory purity. As shown in Figure 2C, the contaminating product (retention time 4.9 min) was essentially eliminated by a single cycle of HPLC purification. At the same time, the contaminating dTTP level in DHdTTP was estimated to be a further 100-fold decreased. The overall yield of DHdTTP from dTTP was on the average about 50% in repeated experiments. Although DHdTTP was purified to apparent homogeneity by the criterion of anion-exchange HPLC, we found that DHdTTP was a mixture of C5 diastereoisomers, i.e., (5*S*)- and (5*R*)-5,6-dihydrothymidine 5′-triphosphates (see Alkaline Phosphatase Digestion of Modified Nucleotides).

Figure 3 shows the HPLC elution profiles of dTTP-GLY before (A) and after (B) two cycles of HPLC purification. dTTP-GLY was eluted at a retention time of 18.2 min under the present HPLC conditions. The final assignment of the structure of dTTP-GLY was achieved by the conversion of this product to *cis*-thymidine glycol by alkaline phosphatase and the positive reaction of its alkali-hydrolyzed product toward pDAB reagent (see below). In contrast to DHdTTP, significant numbers of by products (retention times 5.4, 6.1, 10.8, 12.7, 21.9, and 23.6 min) were produced during the reaction (Figure 3A). These byproducts showed extremely weak detector response with the monitoring wavelength at 260 nm, suggesting the loss of chromophore. The retention time (21.0 min) of the peak adjacent to dTTP-GLY was identical with that of dTTP (see also Figure 2A). However, the possibility that this peak was dTTP was eliminated due to its lack of UV absorption at 260 nm. It is inferred from both the loss of chromophore and the retention times that products with retention times of 21.0 and 23.6 min are triphosphate products with thymine ring degradation, while those with retention times of 5.4 and 6.1 min are mono- or diphosphate products. For each cycle of HPLC, dTTP-GLY was about 100-fold purified with respect to the contamination of the adjacent peak (retention time 21.0 min). Thus, after two cycles of HPLC purification, the largest possible cross-contamination of the adjacent peak in dTTP-GLY was reduced to a level of 1 part per $10^4$. The overall yield of dTTP-GLY from dTTP was on the average about 20% in the repeated experiments.

*Alkaline Phosphatase Digestion of Modified Nucleotides.* HPLC-purified DHdTTP and dTTP-GLY were converted to the corresponding nucleosides with alkaline phosphatase (see Materials and Methods). The products were analyzed by reverse-phase HPLC. After alkaline phosphatase treatment of DHdTTP, two products with retention times of 13.5 and 15.7 min were detected in the HPLC analysis (data not shown). These products were identified as (5*S*)-(−)- (13.5 min) and (5*R*)-(+)-5,6-dihydrothymidine (15.7 min) by using markers prepared by catalytic hydrogenation of thymidine. Separation of the markers is shown in Figure 4A. Alkaline phosphatase treatment of dTTP-GLY gave a single product with a retention time of 6.5 min (data not shown), which was assigned to *cis*-thymidine glycol by using an authentic marker.

*Alkali Hydrolysis of DHdTTP and dTTP-GLY.* Alkali-hydrolyzed products of DHdTTP and dTTP-GLY at pH 12 showed positive reaction toward pDAB reagent, which is specific to the ureido structure (Fink et al., 1956). Yellow color developed immediately after the reagent was sprayed. These results are consistent with the fact that 5,6-dihydrothymine and thymine glycol undergo alkali-catalyzed ring opening (Figure 1) to give β-ureidoisobutyric acid (Kondo & Witkop, 1968) and urea (Jensen et al., 1965; Ide et al., 1985), respectively.

*Ability of Modified Nucleotides To Serve as Substrates for DNA Polymerase.* M13mp11 single-stranded DNA was primed with a 17-mer, and the primer was elongated by *E. coli* DNA polymerase I Klenow fragment in the presence of three normal nucleotides (dATP, dGTP, dCTP, 10 μM) and dTTP or a modified nucleotide (1, 10, or 100 μM). If

Table I: Percent Ratio of C5 Diastereoisomers of Dihydrothymidine in [$^3$H]DHdTTP and in DNA Prepared by DNA Polymerase I Catalyzed Incorporation of [$^3$H]DHdTTP

| | S isomer (%) | R isomer (%) |
|---|---|---|
| DHdTTP | 79 (31 263)[a] | 21 (8303)[a] |
| DNA | 73 (24 848)[a] | 27 (8993)[a] |

[a] Actual count (cpm) by HPLC analysis.

DHdTTP or dTTP-GLY can replace dTTP, elongation of the primer should occur in this system and the extent of the primer elongation as measured by [$^3$H]dCTP incorporation should reflect the rate of incorporation of the modified nucleotide. Indeed, significant elongation of the primer was observed in the presence of 1 or 10 $\mu$M DHdTTP, indicating that dHdTTP replaced dTTP. The [$^3$H]dCTP incorporation was approximately a linear function of incubation time within the reaction time (data not shown). As was expected, the slopes obtained with 1 and 10 $\mu$M DHdTTP were different (see below). In a series of experiments the intrinsic value of [$^3$H]dCTP incorporation in the presence of dTTP or a modified nucleotide was corrected by subtraction of the background obtained with three normal nucleotides alone (dATP, dGTP, and dCTP). The slopes of the time course plot of [$^3$H]dCTP incorporation, which represents the relative rates of DHdTTP incorporation, were calculated as 79 (1 $\mu$M) and 703 (10 $\mu$M) cpm/min. These values are based on 2 $\mu$L of assayed reaction mixture and are the average of three data points (for details see Materials and Methods). These slopes are ~10–25-fold lower than the corresponding values [1967 (1 $\mu$M) and 7300 (10 $\mu$M) cpm/min] for dTTP obtained in control experiments. On the basis of these results, the rate of incorporation of DHdTTP was estimated to be ~10–25-fold slower than that of dTTP. In contrast, dTTP-GLY did not replace dTTP and no elongation of the primer was observed. Increasing the dTTP-GLY concentration up to 100 $\mu$M in the reaction mix did not affect the results.

*Assay of Diastereoisomers of DHdTTP Incorporated into DNA.* Since DHdTTP was incorporated into DNA and DHdTTP was a mixture of C5 diastereoisomers, we checked the stereospecificity of DHdTTP incorporation into DNA. For this purpose, activated salmon testes DNA was used as a substrate for DNA polymerase I in the presence of normal dNTPs and [$^3$H]DHdTTP (*S* and *R* mixture). The DNA containing [$^3$H]dihydrothymine was then enzymatically digested to nucleosides, and the amounts of the $^3$H-labeled isomers were analyzed by HPLC. The results of the HPLC analysis are shown in Figure 4. The elution profile of the markers added to the digested sample is shown in Figure 4A. For both digested DNA and DHdTTP, the radioactivity was detected only at the peaks of the *S* and *R* isomers of dihydrothymidine (Figure 4B). Moreover, the ratio of the *S* and *R* isomers incorporated into DNA (*S*:*R* = 73:27) was essentially the same as that of DHdTTP (*S*:*R* = 79:21) (Table I).

## Discussion

The active species ($^{\bullet}$OH, H$^{\bullet}$, $e^-_{aq}$) produced by radiolysis of water attack predominantly the base moieties of DNA (Cerutti, 1975; Scholes, 1976, 1978). In the radiolysis of pyrimidines, which has been well characterized relative to that of purines, both thymine and cytosine undergo saturation of the C5–C6 double bond by OH and H groups (Téoule & Cadet, 1978). Although the yields of individual radiolysis products can be altered by adding appropriate radical scavengers or second solutes (Wada et al., 1982a,b; Nishimoto et al., 1983a,b), the introduction of a unique product into DNA by use of these methods seems to be difficult. Chemical treatment of DNA can sometimes be more specific with respect to base modifications introduced than can radiation. But the number of chemical reagents currently available is limited, and most of these reagents produce byproducts and/or strand breaks (Hayes et al., unpublished results; H. Ide and S. S. Wallace, unpublished results). For this reason it seemed appropriate to look for alternative approaches to introduce unique damages into DNA. In this study we have found that 5,6-dihydrothymine, which is a major radiolysis product produced in DNA under anoxic conditions (Téoule et al., 1978; Dizdaroglu, 1986; Furlong et al., 1986), is specifically introduced into DNA by in vitro DNA synthesis catalyzed by DNA polymerase in the presence of DHdTTP. We also found that both C5 diastereoisomers of DHdTTP were incorporated into DNA with an efficiency dependent on their concentrations in the nucleotide pool. This result suggests that the diastereoisomers of DHdTTP (or their monophosphates after incorporation) are not discriminated by either the polymerase activity or the proofreading (3′→5′ exonuclease) activity associated with *E. coli* DNA polymerase I. Recently, Furlong et al. (1986) found that one diastereoisomer of dihydrothymidine is preferentially produced by $\gamma$-irradiation of native duplex DNA and cellular DNA. Since the present results suggest that both isomers of dihydrothymidine are equally stable in double-helical DNA, it is likely that the stereospecificity of the $\gamma$-ray-induced isomer formation in the double helix is primarily due to the stereospecific reaction of the precursor molecule (radical intermediate or thymidine) rather than the stability of the final product in the double helix.

Although the loss of planarity due to saturation of the C5–C6 double bond is a common feature for both DHdTTP and dTTP-GLY, surprisingly, only DHdTTP was incorporated into DNA by the polymerase reaction. Since DHdTTP and dTTP-GLY are both expected to have affinity to the triphosphate binding site of DNA polymerase I [the affinity to the site is primarily determined by the triphosphate group (Englund et al., 1969)], the discrimination step may be the stabilization of the inserted nucleotide by stacking and base-pairing interactions. Thus it seems reasonable to assume that dihydrothymine does not produce significant disorder in either the primer terminus or the helical structure of the DNA molecule. Recently, we (Ide et al., 1985) and others (Rouet & Essigman, 1985; Hayes & LeClerc, 1986; Clark & Beardsley, 1986) have found that thymine glycol present in the DNA template retains its coding properties; that is, it can form proper base pairing. Thus the major factor leading to the destabilization of the inserted dTTP-GLY seems to be not improper base pairing but disturbance of the stacking interaction with the adjacent base.

So far as we know, DHdTTP is the first example of polymerase-catalyzed incorporation into DNA of a nucleotide that does not have a coplanar base. By the use of DNA sequencing techniques, we have found that DHdTTP replaced only dTTP of the four normal deoxyribonucleoside triphosphates in the polymerase-catalyzed primer elongation, while dTTP-GLY replaced none of them. The details of these results, including the sequence specificity of DHdTTP incorporation, will be published elsewhere (Ide & Wallace, unpublished results).

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**Registry No.** (*S*)-DHdTTP, 106094-51-3; (*R*)-DHdTTP, 106094-55-7; dTTP-GLY, 106094-52-4; dTTP, 365-08-2; DNA

polymerase, 9012-90-2; deoxyribosylurea triphosphate, 106094-54-6; deoxyribosylureidoisobutyric acid triphosphate, 106094-53-5.

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