

5-Hydroxypyrimidine deoxynucleoside triphosphates are more efficiently incorporated into DNA by exonuclease-free Klenow fragment than 8-oxopurine deoxynucleoside triphosphates

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ABSTRACT

Recent studies with 8-oxodeoxyguanosine triphosphate (8-oxodGTP) have suggested that incorporation of oxidized nucleotides from the precursor pool into DNA may have deleterious effects. Here we show that 5-hydroxydeoxycytosine triphosphate (5-OHdCTP) and 5-hydroxydeoxyuridine triphosphate (5-OHdUTP) are more efficient substrates than 8-oxodGTP for *Escherichia coli* DNA polymerase I Klenow fragment lacking proofreading activity, while 8-oxodeoxyadenosine triphosphate (8-oxodATP) is much less efficient. Furthermore, like 8-oxodGTP, 5-OHdCTP can mispair with dA in DNA but with lower efficiency. Since the 5-hydroxypyrimidines are present in normal and oxidized cellular DNA in amounts similar to the 8-oxopurines, these data suggest that enzymatic mechanisms might exist for removing them from the DNA precursor pools.

INTRODUCTION

Free radicals produce a broad spectrum of damages to cellular DNA molecules (1–3), including a number of lesions that do not block DNA replication and are potentially mutagenic (4–6). Recent interest has focused on 8-oxoguanine, a non-blocking lesion frequently found in normal cellular DNA (7,8) as well as in DNA damaged by a variety of oxidizing agents (3,9,10). 8-Oxoguanine in DNA has been shown to form a stable interaction with both cytosine and adenine (5), the latter leading to G → T transversion mutations (11–13). 8-Oxoguanine opposite cytosine is removed from DNA by formamidopyrimidine (FAPY) DNA glycosylase (14). Adenine is removed from A-8-oxoG mispairs by a DNA glycosylase that is the product of the *mutY* gene (15). Lack of either FAPY DNA glycosylase (*mutM* gene product) or MutY confers a mutator phenotype to *Escherichia coli* cells (for a review see 16). The structurally related 8-oxoadenine at one particular site in DNA is not

mutagenic (17) and thus correctly pairs with T. *In vitro*, however, 8-oxoadenine has been shown to mispair with G (18).

Frequently observed point mutations produced by oxidizing agents (19,20) and ionizing radiation (21–24), especially in free DNA, are C → T transitions implicating cytosine as the target. The major oxidative product of cytosine is cytosine glycol which is unstable, leading to the formation of uracil glycol, 5-hydroxycytosine, and 5-hydroxyuracil (3,25). *In vitro*, 5-hydroxycytosine has been shown to pair primarily with G, but also with A and C (6). 5-Hydroxyuracil pairs primarily with A and in certain sequence contexts, with C (6). Thus the pairing properties of both these lesions could lead to the observed C → T transitions. 5-Hydroxycytosine and 5-hydroxyuracil are recognized *in vitro* by endonuclease III and FAPY DNA-glycosylase (26); 5-hydroxyuracil is also recognized by uracil DNA-glycosylase (26). *Escherichia coli* mutants lacking any of these gene products exhibit a mutator phenotype (16,27,28) although it is not clear whether this phenotype is related to their ability to recognize oxidative products of cytosine.

In addition to possessing enzymes that recognize and remove offending pre-mutagenic lesions from DNA, cells appear to have evolved additional precautions for removing potentially mutagenic oxidized precursors from the metabolic pool. For example, the bacterium *Escherichia coli* (29), as well as human cells (30), possess an enzyme which hydrolyzes 8-oxodGTP to 8-oxodGMP removing it as a precursor for DNA synthesis. The importance of this step is underscored by the fact that *E.coli* cells lacking this nucleoside triphosphatase (MutT) exhibit a mutator phenotype (31).

The implication of the above studies is that oxidized nucleotides are formed at such a significant rate in the cellular pools that they must be removed to reduce the probability of mutation induction. However, little is known about either the steady state levels of oxidized nucleotides in the nucleotide pools or the rate of formation of oxidized precursors by exogenous agents. However, the 8-oxopurines are present in high levels in DNA

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isolated from normal cells (32) as well as in DNA isolated from cells treated with oxidative agents (9,33) or ionizing radiation (9,34). In fact, 8-oxoguanine is currently the benchmark for oxidative DNA damage (35). Similar high levels of the cytosine products are also found in DNA under these same circumstances (25,33,34).

8-OxodGTP has been shown to be a substrate for the α subunit of the *E. coli* DNA polymerase holoenzyme (29) and *E. coli* Klenow fragment lacking proofreading exonuclease (13) under conditions where 8-oxodGTP fully substituted for dGTP or was in 10 to 100-fold excess over the normal dNTPs, respectively. It has recently been demonstrated (36) that 8-oxo-dGTP is misincorporated opposite A *in vitro* by Klenow, T4, *Thermus thermophilus*, and γ polymerases, as well as by HeLa cell extracts, where 8-oxodGTP was present at equimolar concentrations to the normal dNTPs. 5-OHdCTP and 5-OHdUTP are also substrates for *E. coli* DNA polymerase I Klenow fragment and 5-OHdCTP, like 8-oxodGTP, can be misincorporated opposite A (6). In the present study, we compare the relative efficiencies of incorporation of the 5-hydroxypyrimidine to the 8-oxopurine deoxynucleoside triphosphates using *E. coli* DNA polymerase I Klenow fragment lacking proofreading activity. The results show that the 5-hydroxypyrimidine deoxynucleoside triphosphates are significantly better substrates for DNA polymerase than the 8-oxopurines; moreover, the efficiency of misincorporation of 5-OHdCTP opposite dA is only five-fold lower than the efficiency of misincorporation of 8-oxodGTP opposite dA.

MATERIALS AND METHODS

Chemicals, enzymes and DNA

1,1'-Carbonyldiimidazole, Dowex 50 \times 8-100, and tributylamine were obtained from Aldrich; DEAE Sephadex A-25 was obtained from Sigma; γ -³²P-ATP (>5000 Ci/mmmole, 10 mCi/ml) was obtained from Amersham; 2'-deoxynucleoside triphosphates were purchased from Pharmacia; 2',3'-dideoxynucleoside triphosphates were obtained from US Biochemicals. All enzymes, M13 mp18 DNA, primers M13 (-40) GTTTTCCAGTCACGAC and M13 UNIVERSAL (-20) GTAAAACGACGGCCAGT were purchased from US Biochemicals.

Oligodeoxyribonucleotides

All oligonucleotides were synthesized by the standard phosphoramidite method on an ABI 380A DNA synthesizer (Department of Microbiology and Molecular Genetics, University of Vermont).

The oligonucleotides were purified by Mono Q (Pharmacia) anion-exchange chromatography on a Milton Roy HPLC system. The Mono Q column (HR 5/5) was equilibrated with buffer A (0.18 M NaCl, 0.01 M NaOH) at 1 ml/min. Crude deprotected oligonucleotides were loaded on the column and then eluted with a linear gradient of 100% buffer A to 50% buffer A/ 50% buffer B (buffer B — 0.9 M NaCl, 0.01 M NaOH) over 60 minutes. After purification, oligonucleotides were concentrated with the Speed Vac (Savant) and desalted by gel-filtration on a NEP-5 column (Pharmacia) using water as an eluent.

The oligonucleotides were 5'-³²P-labeled with γ -³²P-ATP using T4 polynucleotide kinase following standard procedures. Labeled oligonucleotides were further purified using a NENSORB 20 Nucleic Acids Purification Cartridge (Du Pont). To obtain the desired final specific radioactivity, labeled

oligonucleotides were combined with the appropriate cold oligonucleotides.

Synthesis of 8-oxodGTP

7,8-dihydro-8-oxo-2'-deoxyguanosine-5'-triphosphate (8-oxodGTP) was prepared from dGTP using the modification of the method of Kasai and Nishimura (37). 25 mg of dGTP (sodium salt; 44 μ mole) was dissolved in 9.8 ml of 0.14 M sodium phosphate buffer (pH 6.8) in 25 ml pear-shaped flask and 1.76 ml of 0.1 M ascorbic acid, 0.82 ml of 0.1 M EDTA and 0.164 ml of 0.1 M FeSO₄ were added. The reaction mixture was incubated for 3 hours at 37°C in the dark with constant bubbling of oxygen through the solution. Following this incubation, the reaction mixture was diluted to 250 ml with water and loaded on to a DEAE Sephadex A-25 column (80 ml, HCO₃⁻ form). The fraction containing triphosphates was eluted from the column with a linear gradient of triethylammonium bicarbonate (TEAB) pH 7.5 (5 mM to 1 M). The optical density of the eluate was monitored at 260 and 300 nm. The second part of the peak of triphosphates, adsorbing at 300 nm, was pooled and evaporated several times with 50% ethanol to remove TEAB. 8-oxodGTP was purified twice by HPLC on a C18 column (0.4 \times 25 cm) using 10 mM sodium acetate, 3.5 mM sodium citrate buffer pH 5.1 containing 5% of methanol as the eluent. The elution peak corresponding to 8-oxodGTP was identified by its UV spectrum and oxidation potential value. The peak of 8-oxodGTP was concentrated and desalted on the C18 column using water as an eluent. The yield of 8-oxodGTP was about 5–8%. The molar absorptivity [ϵ = 12,300 (λ_{\max} = 245 nm) and ϵ = 10,300 (λ_{\max} = 293 nm)] (37) was used to calculate the amount of 8-oxodGTP.

Synthesis of 8-oxodATP

7,8-dihydro-8-oxo-2'-deoxyadenosine-5'-triphosphate (8-oxodATP) was prepared from 8-oxodAMP following the method of Hoard & Ott (38). 8-oxodAMP was previously synthesized in the laboratory by Hiroshi Ide by the method described (39,40).

A water solution (~0.3 ml) containing 100 O.D.₂₆₉ (~6.7 μ mole) of 8-oxodAMP (sodium salt) was loaded on to a Dowex 50 \times 8 (pyridinium) column (1 ml) and washed out with ~8 ml of water. 2 μ l (~7 μ mole) of tributylamine was added, the solution was concentrated under vacuum and the residue was dried by repeated addition and evaporation of anhydrous pyridine followed by addition and evaporation of two 1 ml portions of N,N-dimethylformamide (DMF). The residue was dissolved in 100 μ l of dry DMF and 1,1'-carbonyldiimidazole (5.5 mg, 34 μ mole) was added in 70 ml of DMF. The mixture was tightly stoppered and held at room temperature for 4 hours and then treated with anhydrous methanol (2 μ l, 53 μ mole). After 30 min at room temperature, tributylammonium pyrophosphate (35 μ mole) in 350 μ l of DMF was added with vigorous mixing, and the stoppered mixture was held at room temperature for 1 day. The precipitate (imidazolium pyrophosphate) was separated by centrifugation and washed with DMF (two 100 μ l portions). The supernatant was evaporated under vacuum to dryness. The residue was chromatographed on a DEAE sephadex A-25 column (~10 ml) with a linear gradient of TEAB buffer (5 mM to 1 M in 400 ml), pH 7.5. Appropriate fractions were pooled and evaporated several times under vacuum with 50% ethanol to remove TEAB. 8-oxodATP was finally purified on a Mono Q column using a linear gradient of NaCl (5 mM to 0.9 M) in 20 mM Tris-HCl

buffer pH 7.5. The peak of 8-oxodATP was collected, diluted about 5 times with water, and loaded on to a 0.5 ml column with DEAE Sephadex A-25 (HCO_3^-), washed with 0.1 M ammonium bicarbonate and eluted from the column with 0.6 M ammonium bicarbonate. Ammonium bicarbonate was removed by multiple evaporations under vacuum with 50% ethanol at 50°C. The yield of 8-oxodATP was 25–30%. The molar absorptivity [$\epsilon = 15,000$ ($\lambda_{\text{max}} = 269$ nm)] (41) was used to calculate the amount of 8-oxodATP.

Synthesis of 5-OHdCTP and 5-OHdUTP

5-Hydroxy-2'-deoxycytidine 5'-triphosphate (5-OHdCTP) and 5-hydroxy-2'-deoxyuridine 5'-triphosphate (5-OHdUTP) were synthesized as described (6).

DNA polymerase reaction using modified dNTPs

The primer, $^{32}\text{pGTAAAACGACGGCCAGT}$ or $^{32}\text{pGTTTTCCCAGTCACGAC}$, annealed to M13 mp18 DNA, was extended using 0.2 unit of Klenow fragment lacking proofreading activity (Kf Exo⁻). The reaction mixture (6 μl) contained 15 mM Tris-HCl pH 7.5, 7.5 mM MgCl_2 , 30 mM NaCl, 4 mM DTT (buffer 'P'), 20 nM of primed DNA and all four combinations (dA,dT,dC; dG,dT,dC; dG,dA,dC and dG,dA,dT, 50 μM each) of three normal dNTPs. DNA polymerase reactions were incubated at 37°C for 15 min in the absence or presence of 50 μM modified dNTPs (8-oxodGTP, 8-oxodATP, 5-OHdCTP or 5-OHdUTP).

Dideoxy sequencing reactions

Dideoxy sequencing reactions, using the exonuclease free version of phage T7 DNA polymerase (Sequenase Ver 2.0), were performed following recommendations of the supplier (US Biochemicals).

DNA polymerase reactions

The reaction mixture (6 ml) was prepared by adding 3.5 μl of a solution containing 5' ^{32}P -labeled primer-template complex, exonuclease free Klenow fragment (Kf Exo⁻) and the buffer to 2.5 μl of water solution containing dNTPs. The final mixture contained 50 nM of primer-template complex, 50 μM of normal dNTP, 15 mM Tris-HCl pH 7.5, 7.5 mM MgCl_2 , 20 mM NaCl, 1–1.5 nM Kf Exo⁻ (0.02–0.03 unit) and various concentrations of the modified dNTP. For 8-oxodGTP and 8-oxodATP, the concentration range was 10–200 μM ; for 5-OHdCTP, 5-OHdUTP and normal dNTPs (to be incorporated opposite position 3 of the template, Figure 5), 0.05–10 μM . The mixture was incubated for 4 min at 4°C and was stopped by the addition of 4 ml of loading buffer containing 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 20 mM EDTA.

The reaction conditions for the kinetic experiments were similar except the reaction times varied between 0.5 and 15 min.

Electrophoresis

All enzymatic reactions were terminated by the addition of an equal volume of loading buffer. Immediately before loading on to the gel, the samples were denatured at 75°C for 2 min. Reaction products were analyzed by electrophoresis on 0.4 mm thick 13% polyacrylamide gels containing 8 M urea. The gels were electrophoresed in 50 mM Tris-borate, 2 mM EDTA buffer, pH 8.3 for 2–4 h at 3000V, dried under vacuum, and

exposed to X-ray film. The radioactivity in the bands corresponding to the products of the enzymatic reactions was analyzed using a Model GS-250 Molecular Imager System (Bio Rad).

RESULTS

Synthesis of modified dNTPs

8-oxodATP was prepared from 8-oxodAMP by the imidazolid activation of the phosphomonoester group, followed by treatment with excess tetrabutylammonium pyrophosphate (39). 8-oxodATP was purified by ion-exchange HPLC using a Mono Q column. The conversion of 8-oxodAMP to 8-oxodATP, estimated on the basis of HPLC data, was about 45–50%.

8-oxodGTP was synthesized from dGTP by hydroxylation at position C-8 with ascorbic acid in the presence of oxygen (O_2) and Fe^{2+} ions (37) and purified by ion-exchange chromatography on DEAE-Sephadex A-25 and twice by HPLC on a C18 column. Under the conditions used, the retention time for dGTP and 8-oxodGTP was 3.2 and 4.5 minutes, respectively. The elution peak corresponding to 8-oxodGTP was identified by its UV spectrum and oxidation potential value. Based on HPLC data, the conversion of dGTP to 8-oxodGTP was estimated as 10%.

5-OHdCTP and 5-OHdUTP were prepared from dCTP and dUTP, correspondingly, by the action of bromine in aqueous medium followed by 2,4,6-collidine (or pyridine) catalyzed hydrolysis (6).

For DNA polymerase reactions, only freshly prepared modified dNTPs containing less than 5% of the corresponding dNDPs were used.

The specificity of incorporation of 8-oxodGTP, 8-oxodATP, 5-OHdCTP and 5-OHdUTP during DNA synthesis

To study whether 8-oxodGTP, 8-oxodATP, 5-OHdCTP and 5-OHdUTP could replace normal dNTPs as substrates for DNA polymerase, we used a 'three dNTP' assay (42). In this experiment, the primer-template complex was incubated with DNA polymerase in the presence of only three of four dNTPs. Under these conditions ('minus' reaction), the DNA polymerase catalyzing primer elongation pauses opposite each template position complementary to the 'missing' dNTP. Stimulation of the primer extension reaction is observed if in the 'plus' reaction (same three dNTPs plus a modified dNTP), the added modified dNTP is incorporated in place of the missing dNTP.

The exonuclease free Klenow fragment (Kf Exo⁻) catalyzed extension of 17-member $^{32}\text{pGTAAAACGACGGCCAGT}$ or $^{32}\text{pGTTTTCCCAGTCACGAC}$ primers, annealed to M13 mp18 DNA under the conditions of minus and plus reactions, is shown in Figure 1. The extension products were analyzed by high resolution sequencing gels.

8-oxodGTP (Panel A) can replace both dGTP (compare lanes 2 and 3) and dTTP (compare lanes 5 and 6) during DNA synthesis. However, the efficiency of incorporation of 8-oxodGTP in place of dGTP as well as in place of dTTP was much lower than the efficiency of incorporation of dGTP or dTTP (compare lanes 3, 6 and 7). In the presence of equimolar amounts of all four normal dNTPs, polymerization in the presence of 8-oxodGTP was noticeably reduced (compare lanes 7 and 8). A slowdown of replication was observed when the DNA polymerase passed doublets of dC (compare lanes 1 and 3) or dA (compare lanes 4 and 6) in the template, suggesting that

distortion of two 8-oxoG-C or -A pairs occurs thereby reducing elongation.

8-oxodATP was a poor substrate for Kf Exo⁻ (Panel B). Also, 8-oxodATP was incorporated into DNA only in place of dATP (compare lanes 2 and 3) and with a much lower efficiency than normal dATP (compare lanes 3 and 4). As was observed with 8-oxodGTP as a substrate, substantial pausing (or abortion) of synthesis opposite dT doublets in the template (compare lanes 1 and 3) was found with 8-oxodATP again suggesting inefficient extension of two 8-oxodA-dT pairs. In contrast to 8-oxodGTP, however, the presence of 8-oxodATP, did not influence the rate of incorporation of the four normal dNTPs (compare lanes 4 and 5).

Figure 1, Panel C shows the specificity of incorporation of 5-OHdCTP into DNA. A comparison of the extension products showed that 5-OHdCTP replaced both dCTP (compare lanes 5 and 6) and dTTP (compare lanes 2 and 3). However, the efficiency of incorporation of 5-OHdCTP in place of dCTP was much greater than incorporation of either 8-oxodGTP or 8-oxodATP. In contrast to 8-oxodGTP, the presence of 5-OHdCTP did not inhibit incorporation (compare lanes 7 and 8).

Using a similar approach, we found that 5-OHdUTP can be incorporated into DNA (Panel D) but only in place of dTTP (lanes 2 and 3). Incorporation occurred with high efficiency.

A similar specificity of incorporation of 8-oxodGTP, 8-oxodATP, 5-OHdCTP and 5-OHdUTP was found using

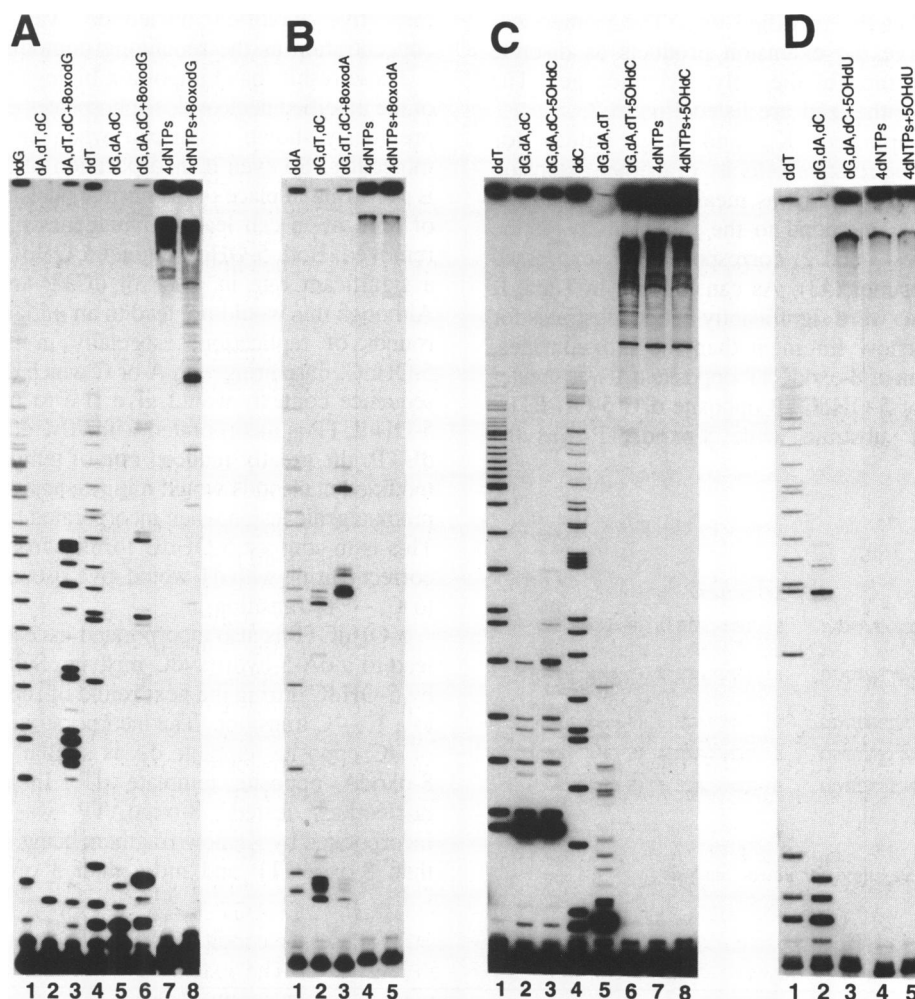


Figure 1. Specificity of incorporation of 8oxodGTP (Panel A), 8oxodATP (Panel B), 5-OHdCTP (Panel C) and 5-OHdUTP (Panel D) catalyzed by *E. coli* DNA polymerase I Klenow fragment (exonuclease free). The extension of ³²P_GTTTTTCCCAGTCACGAC (Panels A and D) or ³²P_GTAAAACGACGGCCAGT (Panels B and C), annealed to M13mp18 DNA, using *E. coli* Kf (Exo⁻) was measured. Panel A: Lanes 2 and 3 represent extension in the absence of dGTP ('-dG' reaction) without and with the addition of 50 μM 8oxodGTP, respectively; lanes 5 and 6 represent the '-dA' reaction without and with the addition of 50 μM 8oxodGTP, respectively; lane 7 shows the extension of the primer in the presence of all four dNTPs (50 μM each); lane 8, same as 7 but containing in addition 50 μM 8oxodGTP. Panel B: Lanes 2 and 3 represent the '-dA' reaction without and with the addition of 50 μM 8oxodATP, respectively; lane 4 shows the extension of the primer in the presence of all four dNTPs (50 μM each); lane 5, same as 4 but containing in addition 50 μM 8oxodATP. Panel C: Lanes 2 and 3 represent the '-dC' reaction without and with the addition of 50 μM 5-OHdCTP, respectively; lanes 5 and 6 represent the '-dT' reaction without and with the addition of 50 μM 5-OHdCTP, respectively; lane 7 shows the extension of the primer in the presence of all four dNTPs (50 μM each); lane 8, same as 7 but containing in addition 50 μM 5-OHdCTP. Panel D: Lanes 2 and 3 represent the '-dT' reaction without and with the addition of 50 μM 5-OHdUTP, respectively; lane 4 shows the extension of the primer in the presence of all four dNTPs (50 μM each); lane 5 same as 4 but containing in addition 50 μM 5-OHdUTP. Lanes 1 and 4 (Panel A), 1 (Panel B), 1 and 4 (Panel C) and 1 (Panel D) are standard dideoxy sequencing reactions using Sequenase.

Sequenase (Ver. 2.0) and Klenow fragment with proofreading activity (data not shown).

Steady state kinetic analysis of 8-oxodGTP, 8-oxodATP, 5-OHdCTP and 5-OHdUTP incorporation

To quantify the insertion of modified dNTPs, a steady state kinetic assay (43) was used. Here we investigated the insertion of 8-oxodG opposite the template dC and dA, 8-oxodA opposite dT, 5-OHdC opposite dG and dA, and 5-OHdU opposite dA using Kf Exo⁻. To determine the kinetic parameters of incorporation, a 16 member primer and a set of 28 member templates were prepared. The primer and templates were designed to place the target site in the template at the third position downstream from the primer. So before reaching the target site, the polymerase must first have incorporated two normal nucleotides and after that the modified one. The primer was 5'-³²P labeled to observe the extension products as discrete bands on the autoradiograph of the polyacrylamide gel. The primer and templates synthesized are listed Figure 2.

To determine the apparent K_m and V_{max} values for incorporation (Table I) the relative velocity of primer extension with each of the modified dNTPs was measured as I_3/I_2 at $t = 4$ min where I_3 and I_2 correspond to the radioactivity of the extension product at sites 3 and 2, correspondingly, expressed as percentage of total primer (43). As can be seen in Table I, the 5-hydroxypyrimidines were significantly better substrates for DNA polymerase I Klenow fragment than the 8-oxopurines, although misincorporation of 8-oxodGTP opposite dA was greater than misincorporation of 5-OHdCTP opposite dA. 5-OHdUTP was the most efficient substrate while 8-oxodATP was the poorest.

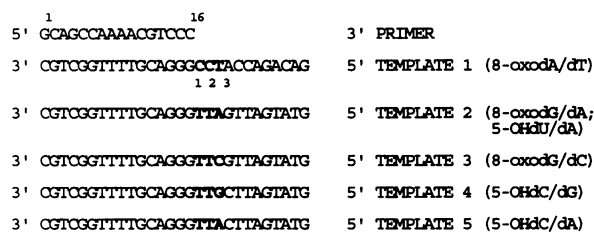


Figure 2. Primer and templates used for kinetic analysis.

Table I.

Template	Substrate	K_m (μ M)	V_{max}	V_{max}/K_m	Relative Efficiency
dG	dCTP	0.2	3.1	15.5	1.0
dG	5-OHdCTP	1.37	3.1	2.26	0.15
dA	dCTP	37	0.05	1.35×10^{-3}	8.7×10^{-5}
dA	5-OHdCTP	27	0.06	2.2×10^{-3}	1.4×10^{-4}
dA	dUTP	0.82	2.8	3.4	1.0
dA	5-OHdUTP	0.07	0.17	2.4	0.71
dC	dGTP	0.1	1.16	11.6	1.0
dC	8-oxodGTP	63	0.32	5.1×10^{-3}	4.4×10^{-4}
dA	8-oxodGTP	58	0.49	8.4×10^{-3}	7.2×10^{-4}
dT	dATP	1.54	3.7	2.4	1.0
dT	8-oxodATP	32	0.1	3.1×10^{-3}	1.3×10^{-4}

DISCUSSION

Both the 8-oxopurine and 5-hydroxypyrimidine deoxynucleoside triphosphates were efficiently incorporated in place of their normal nucleotide analogs by exonuclease free Pol I Klenow fragment when present in an equimolar concentration to the remaining three normal dNTPs. With both 8-oxodGTP and 8-oxodATP, replication was inhibited when two or more of the modified deoxynucleoside triphosphates were incorporated in sequence, suggesting that incorporation of two adjacent 8-oxopurines distorts the DNA thus reducing efficient extension. In contrast, incorporation of two adjacent hydroxypyrimidines did not appear to reduce extension. In addition to substituting for the corresponding normal dNTPs, 8-oxodGTP substitutes for dTTP as has been previously shown, and interestingly, 5-hydroxydCTP also substitutes for dTTP. Here again the respective modified nucleotide was present at the same concentration as the remaining unmodified nucleotides.

These results have important biological implications since all of the modified nucleoside triphosphates examined here have been previously shown to mispair when present in template DNA molecules. So even if incorporation of the modified nucleotide is occurring in place of the normal nucleotide, subsequent rounds of replication can lead to mutagenesis if these lesions are not removed. Both 5-OHdCTP and 5-OHdUTP are incorporated at a significant rate in place of dCTP and dUTP, respectively. Although this would not lead to an initial mispair, in subsequent rounds of replication, especially in the case of unrepaired 5-OHdC, mispairing with A or C which depends on the particular sequence context, would give rise to mutations. Even though 5-OHdUTP is incorporated with 70% efficiency as compared to dUTP, the greatly reduced rate of templated mispairing of this modified nucleotide would mitigate against its being an important premutagenic lesion when incorporated from the nucleotide pool. This is in contrast 5-OHdU formed from dC in DNA. Here its correct pairing with dT would give rise, at a very high efficiency, to C \rightarrow T transitions.

5-OHdCTP is also incorporated instead of dTTP which would lead to a dA-5-hydroxydC mispair. Subsequent correct pairing by 5-OHdC during the next round of replication would give rise to a T \rightarrow C transition. The incorporation of 5-hydroxydC instead of dC opposite template dA is similar to the incorporation of 8-oxodA opposite template dT. In fact, of the modified nucleotides tested, 8-oxodATP was the least efficiently incorporated by Klenow fragment being 3- to 5-fold less efficient than 8-oxodGTP and more than 3 orders of magnitude less

efficient than the 5-hydroxypyrimidines. Since 8-oxodATP is incorporated only in place of its normal nucleotide dA and at extremely low efficiency, it, like 5-OHdUTP, is likely to be a relatively unimportant oxidative lesion when present in the metabolic pools. As was shown with the α subunit of the *E. coli* DNA polymerase holoenzyme (29), the relative incorporation efficiencies by Kf exo⁻ of 8-oxodGTP in place of either dGTP or dTTP are similar and only several fold better than incorporation of 5-OHdCTP in place of dTTP. Thus, if one can extrapolate from the data with Pol I Klenow fragment, the formation of 5-OHdCTP in the precursor pool could lead to mutations. It should be noted however, that the efficiency of incorporation of 8-oxodGTP compared to dGTP by the α subunit of holoenzyme (29) is about two orders of magnitude greater than that observed in these experiments with Klenow fragment. With Klenow fragment, the difference in K_m between dGTP and 8-oxodGTP is about 600-fold, whereas for the α subunit of holoenzyme, it is only about 10-fold. Mutant frequencies produced by incorporation of 8-oxodGTP have also been found to be dependent on the polymerase used (36).

From a biochemical basis, the likelihood of incorporation of 8-oxodGTP into DNA appears to be rather small. The nucleotide pool of dGTP is approximately 120 μ M (44,45). Even if as much as 1% of the pool dGTP is oxidized, this concentration is still well below the K_m even for Pol III holoenzyme. In contrast, if 1% of the pool of dCTP (70 μ M) (44,45), is present as 5-OHdCTP, this concentration is much closer to the K_m of 5-OHdCTP for Klenow fragment and, if the relative comparison holds as was observed for 8-oxodGTP, this could even be above the K_m for the holoenzyme. However, the most compelling evidence that incorporation of oxidized precursors occurs and is relevant comes from the biological data. That is, cells that are missing 8-oxodGTP nucleoside triphosphatase exhibit a substantially higher background mutation frequency than normal cells (16). Accordingly, it might be envisioned that a similar enzyme would be present in *E. coli* to remove oxidized cytosine nucleotides from the precursor pool.

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