Misincorporation of 2′-Deoxyoxanosine 5′-Triphosphate by DNA Polymerases and Its Implication for Mutagenesis†

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ABSTRACT: 2′-Deoxyoxanosine (dOxo) is a novel DNA lesion produced by the reaction of 2′-deoxyguanosine (dGuo) with nitrous acid and nitric oxide [Suzuki, T., Yamaoka, R., Nishi, M., Ide, H., and Makino, K. (1996) *J. Am. Chem. Soc. 118*, 2515−2516]. In this work, 2′-deoxyoxanosine 5′-triphosphate (dOTP) was prepared by nitrous acid treatment of 2′-deoxyguanosine 5′-triphosphate (dGTP), and its incorporation into DNA by DNA polymerases was investigated to elucidate the substrate and mutagenic properties of dOTP. Primed M13mp18 DNA was replicated by *Escherichia coli* DNA polymerase I Klenow fragment (Pol I Kf) in the presence of three normal dNTPs and dOTP or 2′-deoxyxanthosine 5′-triphosphate (dXTP), another major product of reaction of dGTP with nitrous acid and nitric oxide. dOTP substituted for dGTP and to a lesser extent for dATP, while dXTP substituted slightly for dGTP but not for dATP. Neither dOTP nor dXTP substituted for dCTP and dTTP. The similar results were obtained for the incorporation by T7 DNA polymerase deficient in 3′−5′ exonuclease [T7(exo$^-$)]. To quantify the substitution efficiency, kinetic parameters for incorporation of dOTP and dXTP opposite template C or T by Pol I Kf (exo$^-$) were determined and compared with those for dGTP using oligodeoxynucleotide templates. Incorporation efficiencies ($f = V_{max}/K_m$) of dOTP ($f = 0.28\%$ min^{-1} μM^{-1}) and dXTP ($f = 0.10\%$ min^{-1} μM^{-1}) opposite template C were much lower than that of dGTP ($f = 1506\%$ min^{-1} μM^{-1}). Frequencies of mutagenic incorporation of dOTP opposite template T were dependent on the nearest neighbor base pairs, and 1.6−3.9-fold higher than those for dGTP with the nearest neighbors containing G·C pairs. dXTP was not incorporated opposite template T with all four nearest neighbors. These data suggest that formation of dOTP, but not dXTP, from dGTP with nitrous acid or nitric oxide in the intracellular nucleotide pool would result in the elevation of the mutation frequency.

Nitrous acid (HNO_2) reacts with the amino group of guanine (Gua) to result in xanthine (Xan)[1] (*1*, *2*). However, the yield of Xan accounted for only 50% of decomposed Gua when a nucleic acid was treated with nitrous acid (*3*). Subsequently, small amounts of 2-nitroinosine (*4*, *5*) and cross-linked products between Gua moieties (*6*, *7*) were identified as the side reaction products. Recently, the reaction between 2′-deoxyguanosine (dGuo) and HNO_2 was reexamined, and an unknown product was found to be generated at the maximum yield of 21.5%, which corresponded to ca. $^1/_3$ of the total deamination products (*8*). This product was identified as 2′-deoxyoxanosine (dOxo) (Figure 1). dOxo has also been formed in a single-stranded oligodeoxynucleotide and calf thymus DNA by nitrous acid treatment with a comparable yield. Furthermore, dOxo was generated from dGuo in the presence of nitric oxide (NO) at acidic and neutral pHs.

Oxanosine (Oxo) [5-amino-3-β-(D-ribofuranosyl)-3*H*-imidazo[4,5-*d*][1,3]oxazin-7-one], the ribonucleoside counterpart of dOxo, was isolated as a novel antibiotic in 1981 from the culture broth of *Streptomyces capreolus* MG265-CF3 and characterized by X-ray crystallography (*9*, *10*). Oxo exhibits a wide range of biological effects, including in vitro cytotoxicity against HeLa cells and antibacterial activity against *Escherichia coli* K-12 and *Proteus mirabilis* IFM OM-9 (*9*), and induces reversion toward the normal phenotype of K-*ras*-transformed rat kidney cells (*11*). Its analogue, dOxo, has been synthesized from Oxo and found to exhibit stronger antiviral and antineoplastic activities than Oxo (*12*). However, since dOxo had not been regarded as a DNA lesion, no study has been reported for the behavior of dOxo generated in DNA.

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[1] Abbreviations: dOTP, 2′-deoxyoxanosine 5′-triphosphate; dOxo, 2′-deoxyoxanosine; Oxo, oxanosine; Oxa, oxanine (a base moiety of dOTP, dOxo, and Oxo); dXTP, 2′-deoxyxanthosine 5′-triphosphate; dXao, 2′-deoxyxanthosine; Xao, xanthosine; Xan, xanthine; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; Pol I Kf, *Escherichia coli* DNA polymerase I Klenow fragment; Pol I Kf (exo$^-$), Pol I Kf deficient in 3′−5′ exonuclease; T7(exo$^-$), T7 DNA polymerase deficient in 3′−5′ exonuclease (Sequenase, version 2.0); RP-HPLC, reversed-phase HPLC; PAGE, polyacrylamide gel electrophoresis.

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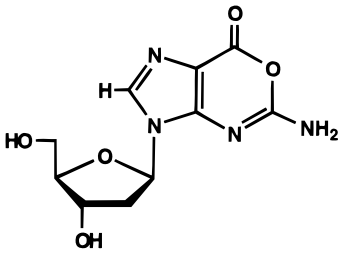

FIGURE 1: Structure of 2′-deoxyoxanosine (dOxo).

Table 1: Oligonucleotide Template−Primers Used in This Study

template/primer	sequence
1	5′-ACCCAGTCACGAA 3′-TGGGTCAGTGCTTCGATAGTTGCTAGC
2	5′-ACCCAGTCACGAG 3′-TGGGTCAGTGCTCTGATAGTTGCTAGC
3	5′-ACCCAGTCACGAC 3′-TGGGTCAGTGCTGTGATAGTTGCTAGC
4	5′-ACCCAGTCACGAA 3′-TGGGTCAGTGCTTTGATAGTTGCTAGC
5	5′-ACCCAGTCACGAT 3′-TGGGTCAGTGCTATGATAGTTGCTAGC

We have recently demonstrated that the N-glycosidic bond of dOxo is as stable as that of dGuo and hydrolyzed 44-fold more slowly than that of 2′-deoxyxanthosine (dXao) (*13*). The melting temperatures of dOxo-containing oligodeoxynucleotide duplexes were much lower than that of the intact duplex containing a normal G·C pair at the same position, but comparable with those of the duplexes containing dXao. These results imply that dOxo generated from dGuo in DNA may have an important and unique role in mutagenic events in cells. In addition, nitrous acid or nitric oxide can also react with 2′-deoxyguanosine 5′-triphosphate (dGTP) in the cellular nucleotide pool, generating 2′-deoxyoxanosine 5′-triphosphate (dOTP). If dOTP is incorporated into DNA in the replication process by DNA polymerase, it can also be mutagenic and cytotoxic. However, there is no available information concerning the dOTP formation by nitrous acid or nitric oxide and its incorporation into DNA.

We report herein the formation of dOTP from dGTP by HNO_2 treatment and the mutagenic misincorporation of dOTP by DNA polymerases during the replication of M13mp18 and oligodeoxynucleotide templates.

MATERIALS AND METHODS

Materials. dGTP used for the preparation of dOTP and dXTP was purchased from Sigma. Highly pure dATP, dGTP, dCTP, and dTTP solutions for DNA polymerase reactions were purchased from Pharmacia. The primer (PrimM13, 5′-GGTTTTCCCAGTCACGA-3′) complementary to positions 6311−6327 in M13mp18 was synthesized on an automated DNA synthesizer and purified by RP-HPLC (*14*, *15*). M13mp18 single-stranded DNA was prepared with the standard procedure (*16*). Oligonucleotides for template−primers 1−5 (Table 1) were obtained from Espec Oligo Service and purified by RP-HPLC. Alkaline phosphatase and *E. coli* DNA polymerase I Klenow fragment (Pol I Kf) were obtained from Toyobo, and T7 DNA polymerase deficient in 3′−5′ exonuclease [Sequenase, version 2.0, T7(exo$^-$)] was from U.S. Biochemicals. Pol I Kf deficient in 3′−5′ exonuclease [Pol I Kf (exo$^-$)] and T4 polynucleotide kinase were from New England Biolabs. [γ-^{32}P]ATP (>185 TBq/mmol) was purchased from Amersham. All other chemicals of reagent grade were purchased from Wako Pure Chemicals or Nacalai Tesque and were used without further purification. Water was purified with a Millipore Milli-QII deionizer. The authentic dOxo and dXao were prepared as described previously (*13*).

RP-HPLC Conditions. The HPLC system consisted of a Shimadzu LC-6A pumping system and a CTO-6A system controller. On-line UV spectra were obtained with a Shimadzu SPD-M6A UV−vis photodiode array detector. For RP-HPLC, a Cosmosil 5C18-MS column (4.6 mm × 150 mm with the particle size being 5 μm, Nacalai Tesque) was used. The eluent was 100 mM triethylammonium bicarbonate (TEAB) buffer (pH 7.0) containing acetonitrile. The acetonitrile concentration was increased from 0 to 20% over the course of 20 min in a linear gradient mode. RP-HPLC analyses were performed at room temperature with a flow rate of 0.9 mL/min.

Quantitative Procedures. The concentrations of the products of reaction of dGTP with nitrous acid were determined by the combination of RP-HPLC peak areas and the molar extinction coefficients (ϵ_{260} for dGTP = 11.5 × 10^3, for dXTP = 7.8 × 10^3, for dOTP = 5.1 × 10^3, and for Xan = 8.5 × 10^3, where values for dOTP and dXTP were assumed to be identical to those of the corresponding nucleosides) (*12*, *16*, *17*).

Reaction Conditions and Preparation of dOTP and dXTP. dGTP (10 mM) was incubated with 100 mM $NaNO_2$ in 3.0 M acetate buffer (pH 3.7) at 37 °C. dOTP and dXTP were separated from the reaction mixture by RP-HPLC with 100 mM TEAB buffer (pH 7.0) as an eluent and then repeatedly lyophilized after addition of H_2O (three times) to remove the buffer.

Alkaline Phosphatase Digestion of dOTP and dXTP. dOTP and dXTP (0.1 mM) were incubated in 100 μL of Tris-HCl buffer (50 mM, pH 8.0) containing alkaline phosphatase (3 units) at 37 °C for 10 min. Aliquots of these solutions (10 μL) were analyzed by RP-HPLC.

DNA Polymerase Reactions Using M13mp18 DNA. Primer PrimM13 was labeled at the 5′-end by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP as described previously (*15*). After incubation at 37 °C for 60 min, unincorporated ATP was removed with a Sep-Pak cartridge (Waters). To examine if dOTP and dXTP could substitute for four normal dNTPs, the minus dNTP reaction (*18*), where one of the four normal dNTPs was missing in the reaction mixture, was performed in the absence or presence of dOTP and dXTP. M13mp18 DNA (0.1 pmol) primed with PrimM13 (template: primer molar ratio of 3:1) in polymerase reaction buffer (20 μL) was replicated by Pol I Kf (0.05 unit) or T7(exo$^-$) (0.5 unit) in the presence of three normal dNTPs (50 μM each) with or without dOTP or dXTP (50 μM) at 37 °C for 30 min. Polymerase reaction buffer consisted of 66 mM Tris-HCl (pH 7.4), 6.6 mM $MgCl_2$, 1.5 mM 2-mercaptoethanol, and 50 μg/mL BSA. The reactions were terminated by the addition of loading buffer (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, and 20 mM EDTA).

Samples were boiled for 3 min and subjected to 8% polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (8 M urea).

DNA Polymerase Reactions Using Oligonucleotide Templates. Primers for template−primers 1−5 (Table 1) were labeled at the 5′-end as described above and annealed to the appropriate templates. Template−primers 1−5 (33 nM) in the polymerase reaction buffer (15 μL) were replicated by Pol I Kf (exo$^-$) (0.015 unit) in the presence of various concentrations of dOTP or dXTP (both 10−100 μM) at 25 °C for 5 min. The reaction was terminated by the addition of the loading buffer. The reactions with dGTP were performed in an essentially similar manner except that the concentration of dGTP was 0.02−0.5 μM and 0.0005 unit of Pol I Kf (exo$^-$) was used. Samples were boiled for 3 min and then separated by 16% PAGE under denaturing conditions (8 M urea). Autoradiography was performed as described previously (*15*). The radioactivity of the primers and extended products was analyzed with Fuji BAS 2000 instrument. To obtain kinetic parameters of the nucleotide incorporation, initial velocities of primer extension (% of the extended primer per minute) were plotted against the dNTP concentrations, and V_{max} (% per minute) and K_m (micromolar) were evaluated by a hyperbolic curve fitting program.

RESULTS

Preparation of dOTP and dXTP. We have previously reported that dOxo is formed efficiently by treating dGuo with nitrous acid (*8*). To prepare the 5′-triphosphate of dOxo (dOTP), a similar nitrous acid treatment was performed with dGTP. The RP-HPLC chromatogram of the reaction mixture obtained after incubation for 2 h is shown in Figure 2A. In addition to the starting dGTP [retention time (t_R) = 12.7 min], three major peaks appeared in the chromatogram. The first eluted peak (t_R = 5.7 min) was xanthine (Xan), which was confirmed by the coincidence of the retention time and UV spectrum with those of the authentic sample. The second (t_R = 11.7 min, λ_{max} = 249 and 280 nm) and fourth (t_R = 13.8 min, λ_{max} = 247 and 289 nm) peaks showed UV spectra characteristic of dXao (λ_{max} = 248 and 278 nm) and dOxo (λ_{max} = 248 and 287 nm), respectively (Figure 2A, insets). These products that had been dephosphorylated by alkaline phosphatase displayed the same retention times and UV spectra as those of authentic dXao and dOxo, respectively (data not shown). Consequently, the second and fourth peaks were assigned to dXTP and dOTP, respectively. The concentrations of the products were quantified by the combination of RP-HPLC peak areas and the molar extinction coefficients. The values were calibrated using the initial dGTP concentration as a standard. The time courses of the formation of the products are shown in Figure 3 along with the conversion of dGTP. The ratio of [dOTP]:[dXTP] was ca. 1:3. Formation of Xan released from dXTP by depurination became apparent after reaction for 1 h. In contrast, oxanine (Oxa, the base moiety of dOTP) was not detected after reaction for up to 3 h. At 2 h, the percentage yields for dOTP and dXTP were 17.1 and 47.0%, respectively (the percentage yields were relative to the initial dGTP concentration). The yield of Xan was 11.5% at 2 h. The dOTP and dXTP isolated from the reaction mixture by RP-HPLC were highly pure as seen from the analytical HPLC profiles (panels B and C of Figure 2) and were used in the DNA polymerase experiments.

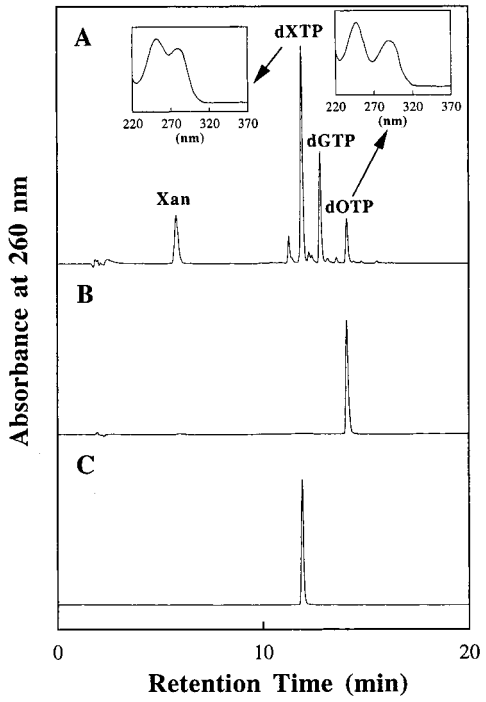

FIGURE 2: RP-HPLC elution profiles of dGTP treated with HNO_2 and purified dOTP and dXTP. Eluents were monitored by UV absorption at 260 nm. The acetonitrile concentration was increased in the linear gradient mode (from 0% at 0 min to 20% at 20 min). (A) The reaction mixture of 1.0 mM 2′-deoxyguanosine 5′-triphosphate (dGTP) and 100 mM $NaNO_2$ incubated in 3.0 M acetate buffer (pH 3.7) for 2 h at 37 °C. Insets are UV spectra of the peaks indicated by the arrows. (B) 2′-Deoxyoxanosine 5′-triphosphate (dOTP) purified by RP-HPLC. (C) 2′-Deoxyxanthosine 5′-triphosphate (dXTP) purified by RP-HPLC.

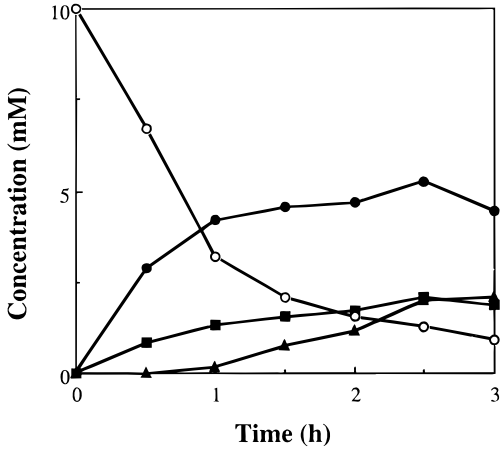

FIGURE 3: Time courses of the concentration change in the products: (○) dGTP, (■) dOTP, (●) dXTP, and (▲) Xan. dGTP (10 mM) was incubated with 100 mM $NaNO_2$ in 3.0 M acetate buffer (pH 3.7) at 37 °C, and the concentration was determined by the combination of the RP-HPLC peak area and the molar extinction coefficient at 260 nm (ϵ_{260} for dGTP = 11.5 × 10^3, for dOTP = 5.1 × 10^3, for dXTP = 7.8 × 10^3, and for Xan = 8.5 × 10^3).

Incorporation of dOTP and dXTP in M13mp18 DNA. To examine whether dOTP and dXTP could substitute for dNTPs (N = A, G, C, or T) during DNA synthesis, the minus dNTP reaction (*18*) was performed. Primed M13mp18 DNA was replicated by Pol I Kf with three normal dNTPs in the presence or absence of dOTP or dXTP. Products were

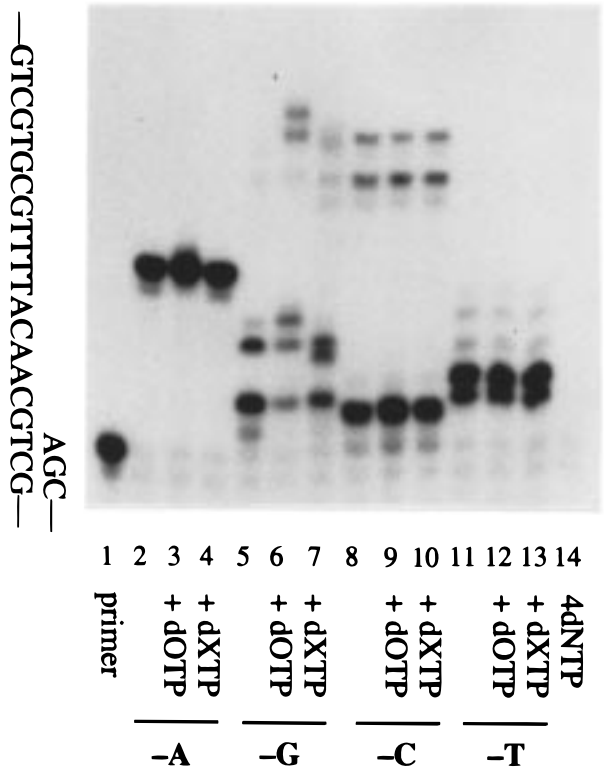

FIGURE 4: Product analysis of −A, −G, −C, and −T reactions with Pol I Kf. Primed M13mp18 DNA was replicated by Pol I Kf at pH 7.0 in the presence of three normal dNTPs (50 μM each) with or without dOTP or dXTP (50 μM) to examine whether dOTP and dXTP could substitute for dNTP missing in the reaction mixture. Products were separated on an 8% denaturing polyacrylamide gel: lane 1, primer; lanes 2−4, −A reaction; lanes 5−7, −G reaction; lanes 8−10, −C reaction; lanes 11−13, −T reaction (lanes 3, 6, 9, and 12 with dOTP and lanes 4, 7, 10, and 13 with dXTP); lane 14, control reaction with four normal dNTPs, where the primer was fully elongated and strong bands appeared at the top of the gel (not shown).

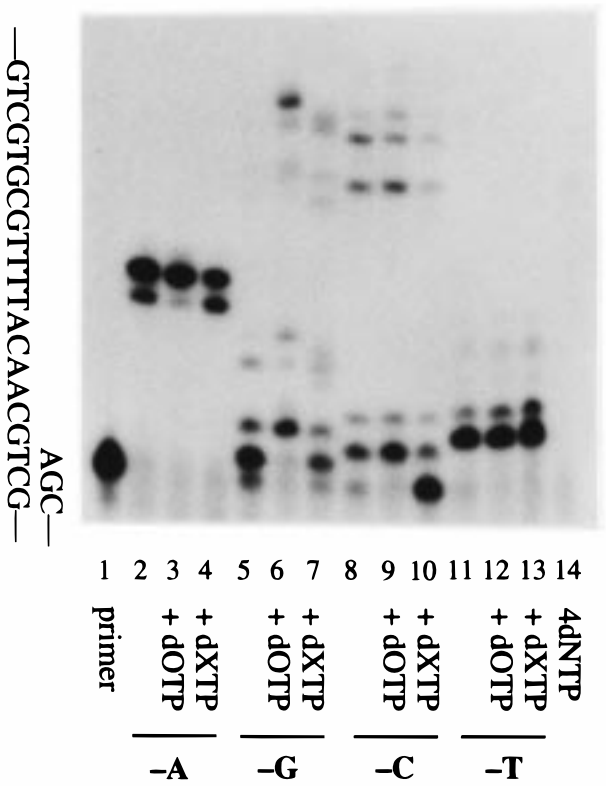

FIGURE 5: Product analysis of −A, −G, −C, and −T reactions with T7(exo$^-$). Except for the type and amount of DNA polymerase used, experimental conditions were the same as those in Figure 4.

analyzed by denaturing PAGE (Figure 4). In the −A reaction, elongation of the primer was terminated in several nucleotides from the primer terminus due to the lack of dATP (lane 2). The faint band just below the major band (the product elongated up to the first T of the four consecutive Ts in the template, lane 2) disappeared with the addition of dOTP (lane 3), implying some substitution of dOTP for the missing dATP. The substitution for dATP was further confirmed by the reactions using T7(exo$^-$) and oligonucleotide templates described below. No extension of the primer over the −A reaction (lane 2) was observed in the presence of dXTP (lane 4). In the −G reaction, the primer was extended over the −G reaction (lane 5) in the presence of dOTP (lane 6) and dXTP (lane 7). The efficiency of the primer elongation with dOTP was higher than that with dXTP. On the other hand, in the −C and −T reactions, elongation over the background was not observed by the addition of dOTP or dXTP under the present reaction conditions (lanes 8−13). Substrate properties of dOTP were also studied using T7 DNA polymerase deficient in 3′−5′ exonuclease [T7(exo$^-$)]. Figure 5 shows the results of product analysis by PAGE. As seen in Figure 5, results essentially the same as those with Pol I Kf were obtained for the incorporation of dOTP and dXTP. In the −C reaction, the primer extension in the presence of dXTP (lane 10) was somewhat less efficient than that of control (lane 8) for an unknown reason. The minus dNTP reactions using M13mp18 DNA revealed that dOTP could substitute dGTP more efficiently than dXTP, and the substitution efficiency for dATP was lower than that for dGTP.

Incorporation of dOTP and dXTP in Oligodeoxynucleotides. To evaluate the substitution efficiency of dOTP and dXTP for dGTP and dATP quantitatively, kinetic parameters for the incorporation of dOTP and dXTP opposite template C or T by Pol I Kf (exo$^-$) were measured using defined oligonucleotide templates (Table 1). The incorporation efficiency opposite template C was determined using template−primer 1, and those opposite template T were measured using template−primers 2−4 containing four different nearest neighbor base pairs adjacent to the incorporation site.

Figure 6A shows a representative gel autoradiogram for the incorporation of dOTP and dXTP opposite template C. Both dOTP and dXTP were incorporated opposite template C, showing that dOTP and dXTP could be substituted for dGTP. It is noted that the product elongated by incorporation of one dXTP (panel A, lanes 6−10) migrated very close to the original primer and was far from that elongated by one dOTP (lanes 1−5). The fast migration of the dXTP product was due to the negative charge in the base moiety of dXao (see the Discussion). By quantifying the radioactivity of the remaining primer and extended products, we calculated the initial velocity of the reaction, and kinetic parameters were determined as described in Materials and Methods. Typical Michaelis−Menten plots are shown in Figure 7, and the parameters (average of two experiments) are summarized in Table 2. K_m values for incorporation of dOTP (94.3 μM) and dXTP (82.7 μM) were roughly 1700-fold higher than that for dGTP (0.051 μM), though the decreases in V_{max} relative to that of intact dGTP were modest (3- and 9-fold).

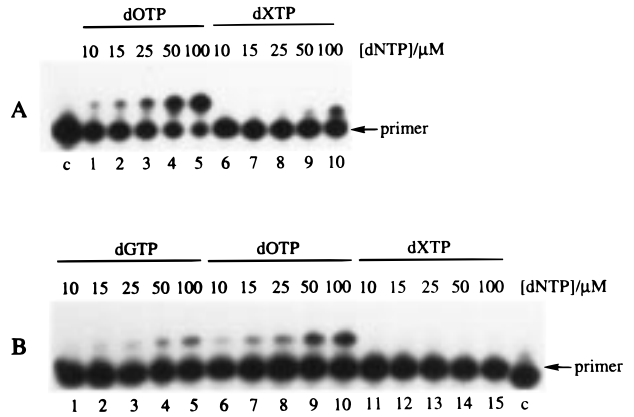

FIGURE 6: Gel electrophoretic analysis of incorporation of dOTP, dXTP, and dGTP opposite C and T by Pol I Kf (exo$^-$) using oligonucleotide templates. Template−primers 1 (A) and 2 (B) were replicated by Pol I Kf (exo$^-$) in the presence of various concentrations of dOTP, dXTP, or dGTP (10−100 μM). Products were separated on a 16% denaturing polyacrylamide gel. (A) Incorporation of dOTP (lanes 1−5) and dXTP (lanes 6−10) opposite template C: lane c, primer without reaction; lanes 1 and 6, 10 μM dOTP and dXTP; lanes 2 and 7, 15 μM dOTP and dXTP; lanes 3 and 8, 25 μM dOTP and dXTP; lanes 4 and 9, 50 μM dOTP and dXTP; and lanes 5 and 10, 100 μM dOTP and dXTP. (B) Incorporation of dGTP (lanes 1−5), dOTP (lanes 6−10), and dXTP (lanes 11−15) opposite template T: lane c, primer without reaction; lanes 1, 6, and 11, 10 μM dGTP, dOTP, and dXTP; lanes 2, 7, and 12, 15 μM dGTP, dOTP, and dXTP; lanes 3, 8, and 13, 25 μM dGTP, dOTP, and dXTP; lanes 4, 9, and 14, 50 μM dGTP, dOTP, and dXTP; and lanes 5, 10, and 15, 100 μM dGTP, dOTP, and dXTP.

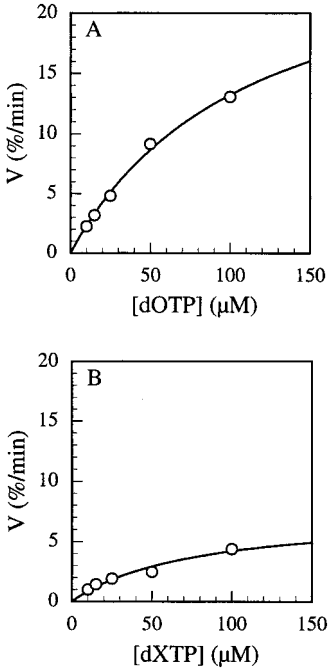

FIGURE 7: Representative Michaelis−Menten plots for incorporation of dOTP and dXTP opposite template C. Initial velocities obtained from the PAGE analysis of the reaction products with template−primer 1 are plotted against the concentration of dOTP (A) and dXTP (B). The curves were generated by a hyperbolic curve fitting program.

Consequently, incorporation efficiencies ($f = V_{max}/K_m$) opposite template C decreased by factors of 5×10^3 (dOTP) and 1.5×10^4 (dXTP) in comparison with that of dGTP.

Figure 6B shows a representative gel autoradiogram for mutagenic incorporation of dGTP, dOTP, and dXTP opposite template T using template−primer 2. Consistent with the results with the M13 DNA template (Figures 4 and 5), incorporation of dXTP opposite template T was not observed with up to 100 μM dXTP (lanes 11−15). Furthermore, dXTP was not incorporated opposite template T with template−primers 3−5 containing different nearest neighbor base pairs (data not shown). Thus, kinetic parameters were obtained for only dOTP and dGTP (Table 3). V_{max} values for dOTP were consistently higher than those for dGTP with all the nearest neighbors. For the nearest neighbor base pairs T·A (template·primer) and A·T, incorporation efficiencies ($f = V_{max}/K_m$) of dOTP were virtually comparable to those of dGTP due to the parallel increases in V_{max}. However, incorporation efficiencies (f) of dOTP were higher than those of dGTP for the nearest neighbors C·G and G·C. The differences between dOTP and dGTP were modest (1.6- and 3.8-fold) but significant.

Table 2: Kinetic Parameters for Incorporation of dGTP, dOTP, and dXTP Opposite Template C[a]

nucleotide	V_{max} (%/min)[b]	K_m (μM)	f (% min^{-1} μM^{-1})[c]
dGTP	76.8 ± 13.6	0.051 ± 0.0006	1506 (1)
dOTP	26.7 ± 1.8	94.3 ± 23.7	0.28 (1.9×10^{-4})
dXTP	8.3 ± 0.6	82.7 ± 4.5	0.10 (6.6×10^{-5})

[a] Average of two experiments. [b] Percent of extended primer per minute with 0.015 unit of Pol I for template−primer 1. The experiment for dGTP was carried out with 0.0005 unit of Pol I, and the V_{max} that was obtained was multiplied by 30 (=0.015/0.0005) to adjust the difference in the amount of Pol I. [c] $f = V_{max}/K_m$ with relative values in parentheses.

Table 3: Kinetic Parameters for Misincorporation of dGTP and dOTP Opposite Template T with Various Nearest Neighbor Base Pairs[a]

template−primer	nucleotide	V_{max}[b] (%/min)	K_m (μM)	f[c] (% min^{-1} μM^{-1})	f_O/f_G[d]
2[e] (C·G)[f]	dGTP	4.0 ± 0.6	78.8 ± 27.4	0.05	
	dOTP	15.5 ± 0.7	80.0 ± 10.1	0.19	3.8
3[e] (G·C)[f]	dGTP	2.8 ± 0.2	57.5 ± 16.4	0.05	
	dOTP	14.5 ± 5.7	178.3 ± 42.7	0.08	1.6
4[e] (T·A)[f]	dGTP	3.9 ± 0.1	28.5 ± 4.5	0.14	
	dOTP	6.9 ± 1.5	45.1 ± 10.0	0.15	1.1
5[e] (A·T)[f]	dGTP	1.5 ± 0.3	13.7 ± 4.5	0.11	
	dOTP	2.3 ± 0.2	20.0 ± 6.1	0.12	1.1

[a] Average of two experiments. [b] Percent of extended primer per minute with 0.015 unit of Pol I. [c] $f = V_{max}/K_m$. [d] $f_O/f_G = f$(dOTP)/f(dGTP) for each nearest neighbor base pair. [e] Template−primer used in the experiment (see Table 1). [f] Bases for the base pair between the template and primer.

DISCUSSION

Formation of dOTP and dXTP. In this study, dOTP and dXTP were synthesized by nitrous acid treatment of dGTP in solution and subsequent separation using RP-HPLC. The yields of dOTP and dXTP after reaction for 2 h were 17.1 and 47.0%, respectively, which were virtually identical with those of dOxo and dXao generated from free dGuo in solution under the same reaction conditions. Moreover, the yields of dOxo and dXao from free dGuo have been found to be comparable to those of dOxo and dXao moieties from dGuo in a single-stranded oligodeoxynucleotide and duplex calf thymus DNA (*8*). Thus, the reactions forming dOxo and dXao moieties with nitrous acid appear to be insensitive to the environment of dGuo whether it is in DNA or in a free nucleoside or nucleotide form.

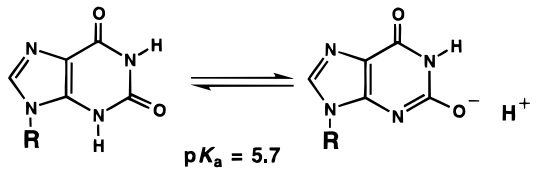

FIGURE 8: Acid−base equilibria for Xao.

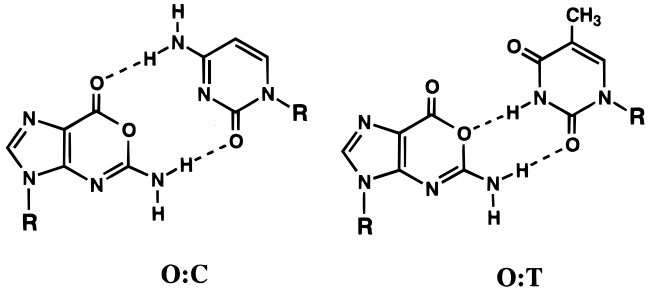

FIGURE 9: Proposed base pairs between dOxo and dCyd, and dOxo and dThd.

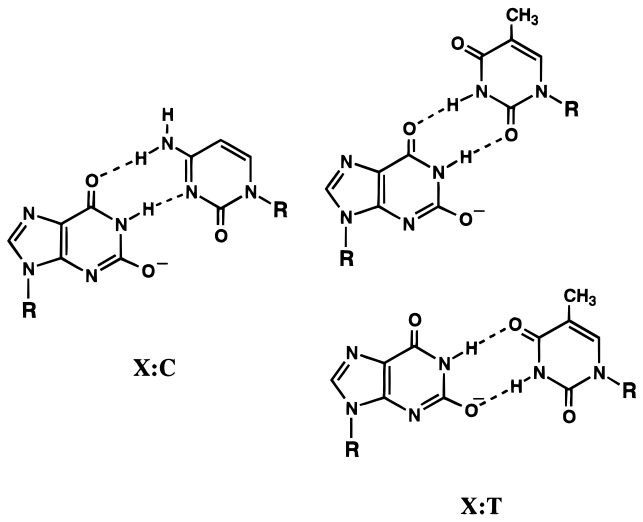

FIGURE 10: Proposed base pairs between dXao and dCyd, and dXao and dThd.

Acid−Base Equilibrium of dOxo and dXao. dOxo has a lactone structure in the base moiety (Oxa). The lactone in the base moiety has a pK_a of 9.4, which can be attributed to the acid−base equilibrium of saponification and relactonization (*13*). Therefore, dOTP exists as the ring-closed form and bears no charge on the base moiety in neutral solution. On the other hand, the base moiety of dXao (Xan) is far more acidic as a heterocycle than normal nucleobases. The pK_a value of xanthosine (Xao) and xanthosine 5′-monophosphate (XMP) is 5.7 (*19*), which is attributed to the acid−base equilibrium of deprotonation of the N3 imino proton (Figure 8). Accordingly, dXTP will exist as the O2 enolate form and bear a negative charge on the base moiety in neutral solution. Oligonucleotides containing dXao migrate faster than those containing dAdo or dGuo at the same positions (*20*). In this study, the primer elongated by the incorporation of dXTP also migrated faster than that containing dOxo at the same position (Figure 6A). The 3′-terminal xanthine moiety in the elongated product was probably deprotonated, whereas 3′-terminal Oxa in the product bore no charge under the present conditions of electrophoresis.

Incorporation of dOTP and dXTP. dOTP was incorporated opposite both template C and T, though the efficiencies ($f = 0.28\%$ min^{-1} μM^{-1} for C, and $f = 0.08-0.19\%$ min^{-1} μM^{-1} for T) were much lower than those for incorporation of normal nucleotides forming canonical Watson−Crick base pairs, for example, dGTP versus template C in this study ($f = 1506\%$ min^{-1} μM^{-1}). With the same nearest neighbor base pairs (template−primers 1 and 4), dOTP was incorporated opposite C ($f = 0.28\%$ min^{-1} μM^{-1}) more efficiently than opposite T ($f = 0.15\%$ min^{-1} μM^{-1}). Presumable base pairing schemes for Oxa (O) with C and T are illustrated in Figure 9. The most likely discrimination principle for the base selection of DNA polymerase is the Watson−Crick structure which is imposed by the structure of a polymerase active site and the hydrogen bonding between paired bases (*21*). When the structure of Oxa is considered, both O·C and O·T pairs can adopt the canonical Watson−Crick geometry in duplex DNA without introducing any steric perturbation. Oxa having the acceptor−acceptor−donor configuration can form two hydrogen bonds with either C or T. In the case of the O·T pair, the ring oxygen atom is used in the hydrogen bonding but not for O·C pair. The ring oxygen atom of Oxa has an sp^3 hybrid orbital. Since it has been shown by X-ray diffraction that the six-membered lactone ring is planar (*10*), the lone pair electrons of the ring oxygen atom of Oxa exist out of the plane. If Oxa in incoming dOTP and template thymine exist in the same plane in the polymerase active site, the hydrogen bonding between the ring oxygen of Oxa and the imino proton of thymine would be weak or even cannot be formed. The lower efficiency for the incorporation of dOTP opposite T than C could be due to the difference in these hydrogen bond-forming abilities between O·C and O·T pairs.

dXTP was incorporated opposite template C but not opposite template T. Bessman et al. (*22*) reported that dXTP was incorporated into DNA with a $^1/_{33}$ efficiency relative to dGTP by a DNA polymerase (Pol I) extracted from *E. coli* (*22*). Recently, Piccirilli et al. (*20*) also reported that the incorporation efficiency of dXTP opposite C in the template was low (ca. 5%; the percentage of the primer elongation) when dGTP was missing from the incubation mixture, and no dXTP was incorporated opposite T (*20*). The results of this study are essentially consistent with these previous studies, although the incorporation efficiency of dXTP opposite C appears to be much lower on the basis of the kinetic parameters of this study. In neutral solution, Xan exists as the 6-keto-2-enolate ion form (*19*). The postulated base pairing schemes for the 6-keto-2-enolate form of Xan with C and T based on the hydrogen bonding capacity are illustrated in Figure 10. The 6-keto-2-enolate form of Xan (X) having the acceptor−donor−acceptor configuration can form two hydrogen bonds with either C or T. The X·C pair configuration fits in Watson−Crick geometry. On the other hand, the X·T pair can assume two configurations, but both only in unfavorable wobble alignments. These alignment differences may explain why dXTP was incorporated opposite C but not opposite T.

Biological Effects of dOTP and dXTP as Damage of dGTP. Since nitric oxide produces dOxo and dXao from dGuo (*8*), dOTP and dXTP are also likely to be produced from dGTP by nitric oxide. Thus, nitrous acid and nitric oxide generated endogenously (*23*, *24*) or originated exogenously will cause the formation of dOTP and dXTP from dGTP in the

intracellular nucleotide pool as well as that of dOxo and dXao from dGuo in DNA. In this work, it has been shown that Oxa generated from Gua has an ambiguous base pairing property since dOTP was incorporated opposite both template C and T, forming O·C and O·T pairs. dGTP, the parental nucleotide of dOTP, is also known to be incorporated opposite template T, forming a G·T mispair. The frequency of O·T pair formation was dependent on the nearest neighbor base pair, and significantly higher than those of G·T formation with the neighbors containing G·C pairs. Therefore, it is likely that formation of dOTP in the cellular dGTP pool will result in an elevated level of mutagenesis as compared with that for dGTP alone.

It has been reported that Xan in DNA is potentially mutagenic (*25*, *26*). This study has shown that dOTP is more efficiently incorporated opposite template C than dXTP (2.8-fold). Moreover, dOTP is also incorporated opposite T of template DNA, while dXTP is not incorporated under the same conditions. Thus, dOTP, if generated in the nucleotide pool, would be introduced into DNA more efficiently and more desultorily than dXTP. Moreover, the N-glycosidic bond of a dOxo moiety in DNA is as stable as that of dGuo, whereas that of dXao is by far more sensitive (44-fold) to hydrolysis (*13*). Since no specific repair enzymes for Xan have been identified so far, it is possible that Xan incorporated in DNA is released spontaneously by nonenzymatic hydrolysis and the resultant abasic sites can be subjected to repair by ubiquitous AP endonucleases (*27*). However, Oxa incorporated in DNA will persist much longer in DNA due to the stable N-glycosidic bond. Thus, the formation of dOTP in the nucleotide pool could result in more serious mutagenic events than that of dXTP.

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