Isolation and Characterization of Diazoate Intermediate upon Nitrous Acid and Nitric Oxide Treatment of 2′-Deoxycytidine†

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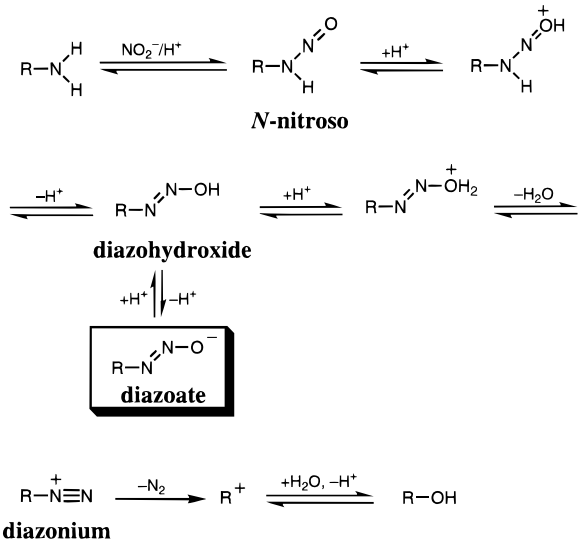
ABSTRACT: The intermediate produced from dCyd by HNO_2 and NO treatments was isolated and characterized. When 10 mM dCyd was treated with 100 mM $NaNO_2$ in 1.0 M acetate buffer (pH 3.7) at 37 °C, a previously unidentified product was formed. By spectrometric measurements, the product was identified as a diazoate derivative of dCyd, 1-(β-D-2′-deoxyribofuranosyl)-2-oxopyrimidine-4-diazoate. The time course of the concentration change of the diazoate showed a profile characteristic of a reaction intermediate, and the maximum yield was 37 μM at the reaction time of 25 min. Up to the reaction time of 10 min, the diazoate concentration was greater than that of dUrd, a deamination product of dCyd. Addition of thiocyanate increased the yield of the diazoate in HNO_2 treatment, whereas addition of ascorbate decreased the yield. When 10 mM dCyd in 100 mM phosphate buffer was treated with NO at 37 °C under aerobic conditions holding the pH (7.2−7.6), the diazoate was also generated. The yield of the diazoate was higher than that of dUrd up to 15 mmol of NO absorption. At pH 3.7 and 37 °C, the diazoate was converted to dUrd with the first-order rate constant $k = 4.8 \times 10^{-4}$ s^{-1} ($t_{1/2} = 24$ min). Under physiological conditions (pH 7.4, 37 °C), however, it was fairly stable ($k = 5.8 \times 10^{-7}$ s^{-1}, $t_{1/2} = 330$ h). In both cases, the diazoate was converted to dUrd exclusively and no other intermediates were detected by HPLC analysis. Uracil-DNA glycosylase did not remove the diazoate residue from an oligodeoxynucleotide containing this damage, [d(T_6DT_5), D = the diazoate]. The T_m value of a duplex containing the diazoate, d(T_6DT_5)·d(A_5GA_6), was much lower than that of a duplex containing a correct C:G base pair, d(T_6CT_5)·d(A_5GA_6). These results show that the diazoate is generated as a stable intermediate in the reactions of dCyd with HNO_2 and NO and that the major product is the diazoate but not dUrd in the initial stage of the reactions. Thus, once formed in vivo, the diazoate persists for long time in DNA and may act as a major cytotoxic and/or genotoxic lesion with biologically relevant doses of HNO_2 and NO.

Nitrous acid (HNO_2) is known to be mutagenic to living cells (*1*−*3*). HNO_2 reacts with bases of nucleic acids such as cytosine (Cyt), adenine (Ade), and guanine (Gua), generating deamination products, uracil (Ura), hypoxanthine (Hyp), and xanthine (Xan), respectively (*4*, *5*). Cyt and Ade are exclusively converted to the corresponding deamination products by HNO_2 (*6*), whereas Gua gives rise to several byproducts including 2-nitro-Hyp (*7*), cross-link products between two Gua residues (*8*), and oxanine (Oxa) (*9*−*11*). Deamination of nucleobases by HNO_2 is thought to proceed via several intermediates including *N*-nitroso, diazohydroxide, diazoate, and diazonium derivatives based on the diazo chemistry of aromatic primary amines (Scheme 1) (*12*, *13*).

Scheme 1



However, despite their importance in mutagenesis, only a diazoate derivative of 9-alkyl Ade formed by isoamyl nitrite has been investigated as a putative intermediate (*13*). Nitric

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oxide (NO) is also mutagenic to cells and generates deaminated nucleobases such as Ura, Hyp, and Xan under aerobic conditions (*14*). Oxa has been recently identified as a byproduct in the reaction of Gua residues with NO (*10*). Although it is proposed that N_2O_3 is the active species of the deamination reaction by the NO treatment under aerobic conditions as well as the HNO_2 treatment (*15*, *16*), the ultimate active species and the reaction pathway of deamination by NO in the presence of O_2 have not been determined (*17*). Since there is no report on byproducts or experimental evidence for intermediates in the reaction of Cyt with HNO_2 or NO, mutagenic events derived from Cyt in DNA treated with HNO_2 or NO have been interpreted solely in terms of the formation of Ura until now.

The deamination of nucleobases also occurs at biologically significant rates in cells via spontaneous hydrolysis. Cyt is deaminated much more rapidly than Ade and Gua under physiological conditions (*18*−*20*). Thus, the specific repair enzyme, uracil-DNA glycosylase, exists abundantly in cells and excises Ura from DNA by cleaving the N-glycosidic bond (*21*, *22*). Ura produced by HNO_2 or NO is also excised by the same enzyme. However, a recent study employing several mutant strains of *Escherichia coli* has shown that a defect in uracil-DNA glycosylase has little impact on mutagenesis by HNO_2 (*23*). Furthermore, similar results were obtained for reversion frequency caused by NO (*24*, *25*). These results suggest that mutagenicity of HNO_2 and NO may not be caused mainly by the formation of Ura from Cyt in DNA. Therefore, other mutation mechanisms not involving Ura need to be elucidated in Cyt-HNO_2 and -NO systems.

In the present study, we have examined the reaction between 2′-deoxycytidine (dCyd) and HNO_2 or NO and found a previously unidentified product, a diazoate derivative of dCyd. The diazoate was not a byproduct but accumulated as a fairly stable precursor of 2′-deoxyuridine (dUrd). We report here identification and characterization of the intermediate.

MATERIALS AND METHODS

Materials. dCyd and dUrd were obtained from Sigma. All other chemicals of reagent grade were purchased from Wako Pure Chemicals or Nacalai Tesque and used without further purification. Nuclease P1 and alkaline phosphatase were obtained from BOEHRINGER, and uracil-DNA glycosylase was from GIBCO. Oligodeoxynucleotides, d(T_6CT_5) and d(A_5GA_6), were obtained from Cruachem and purified by reversed-phase (RP) HPLC.[1] Water was purified with a Millipore Milli-QII deionizer.

HPLC Conditions. The HPLC system consisted of Hitachi L-6000 and L-6200 pumping systems. On-line UV spectra were obtained with a Hitachi L-3000 UV−vis photodiode array detector. For analysis and separation of nucleosides by RP-HPLC, a Cosmosil 5C18-MS octadesylsilane column (4.6 × 150 mm, particle size 5 μm, Nacalai Tesque) was used. The eluent was 100 mM triethylammonium acetate (TEAA) buffer (pH 7.0) containing acetonitrile. The acetonitrile concentration was increased from 0 to 15% for 15 min with a linear gradient mode. RP-HPLC analyses were performed at room temperature and a flow rate of 1.0 mL/min. For oligodeoxynucleotides, a YMC-Pack ODS-A octadesylsilane column (6.0 × 150 mm, particle size 5 μm, YMC) was used. The eluent was 100 mM TEAA buffer (pH 7.0) containing acetonitrile. The acetonitrile concentration was increased from 15 to 25% for 30 min with a linear gradient mode. RP-HPLC analyses were performed at 40 °C and a flow rate of 1.7 mL/min.

Spectrometric Measurements. NMR spectra were measured using a Brucker ARX-500 spectrometer in DMSO-d_6 at 22 °C. The chemical shifts (ppm) were referenced to TMS as an internal standard. The signal assignments were performed by DQF-COSY, ^{1}H-^{13}C HMQC, and ROESY (200 ms). Negative-ion APCI-LC mass spectra were obtained by a Hitachi M-2000 MS system. The sample was directly injected into the MS system by an HPLC pump without column. The MS conditions are as follows: eluent, 100% CH_3CN (isocratic); flow rate, 1 mL/min; vaporization temperature, 300 °C; desolvation temperature, 305 °C; drift voltage, −195 V.

*Spectrometric Data of **1** [1-(β-D-2′-deoxyribofuranosyl)-2-oxopyrimidine-4-diazoate]*. ^{1}H NMR (500 MHz, DMSO-d_6 at 22 °C): δ (ppm/TMS) 7.89 (d, J_{56} = 3.0 Hz, 1H, H-6), 6.73 (d, 1H, H-5), 6.20 (dd, $J_{1'2'}$ = 7.0 Hz, $J_{1'2''}$ = 6.3 Hz, 1H, H-1′), 5.36 (br, 1H, 3′-OH), 5.04 (br, 1H, 5′-OH), 4.23 (ddd, $J_{3'4'}$ = 3.7 Hz, 1H, H-3′), 3.80 (ddd, $J_{4'5'}$ or $J_{4'5''}$ = 3.7 or 4.9 Hz, 1H, H-4′), 3.53 (ABX, $J_{5'5''}$ = 12.0 Hz, 2H, H-5′,5″), 2.18 (ddd, $J_{2''3'}$ = 3.5 Hz, 1H, H-2″), 2.00 (ddd, 1H, H-2′). ^{13}C NMR from ^{1}H-^{13}C HMQC (DMSO-d_6 at 22 °C): δ (ppm/TMS) 139.7 (C-6), 94.8 (C-5), 87.4 (C-4′), 84.9 (C-1′), 70.2 (C-3′), 61.0 (C-5′), 40.3 (C-2′). UV: λ_{max} 285, 310 nm (pH 7). Negative ion APCI-LC/MS (CH_3CN): *m*/*z* 260 (M^- − N_2 + O_2 + H), 255 (M^-), 227 (M^- − N_2).

Preparation of Oligodeoxynucleotides. To obtain oligodeoxynucleotides containing the diazoate and Ura, d(T_6CT_5) was treated by HNO_2 and RP-HPLC separation was performed after neutralization. However, RP-HPLC failed to separate d(T_6DT_5) (*D* = the diazoate) and d(T_6UT_5) (U = Ura) since they comigrated under the present HPLC conditions [retention time (t_R) = 17.1 min]. Thus, the oligodeoxynucleotides were obtained as follows. To obtain d(T_6DT_5), 20 nmol of d(T_6CT_5) was incubated in 100 μL of 1.0 M acetate buffer (pH 3.0) with 100 mM $NaNO_2$ and 500 mM NaSCN at 70 °C for a short period (1.5 min). The sample was subjected to RP-HPLC and the fraction containing d(T_6DT_5) and d(T_6UT_5) was collected and lyophilized. The mixture was incubated with 5 units/mL of uracil-DNA glycosylase in 50 mM Tris-HCl buffer (pH 8.0) at 37 °C for 3 h. This treatment selectively converted d(T_6UT_5) in the mixture to d(T_6abT_5) (ab = abasic site) that was eluted with different retention time (t_R = 12.9 min). d(T_6DT_5) was separated from the reaction mixture by RP-HPLC and lyophilized. The UV spectrum of d(T_6DT_5) exhibited appreciable UV absorbance at wavelengths over 310 nm (A_{310nm}/A_{260nm} = 0.073), which was attributable to the diazoate residue in the oligodeoxynucleotide. To obtain d(T_6UT_5), a similar HNO_2 treatment of d(T_6CT_5) was performed for a prolonged period (90 min). Under these conditions, the product was exclusively d(T_6UT_5) and the formation of d(T_6DT_5) was negligible. Subsequently, d(T_6UT_5) was collected

[1] Abbreviations: RP-HPLC, reversed-phase HPLC; t_R, retention time; APCI LC/MS, atmospheric pressure chemical ionization liquid chromatography mass spectrometry; SPER/NO, spermine/nitric oxide complex; DEA/NO, diethylamine/nitric oxide complex; T_m, melting temperature.

by RP-HPLC, and lyophilized. UV spectrum of d(T_6UT_5) did not exhibit appreciable absorbance over 310 nm. The nucleoside compositions of d(T_6DT_5) and d(T_6UT_5) were confirmed by the enzymatic digestion and subsequent RP-HPLC analysis as follows. Each solution of the oligodeoxynucleotides (1 nmol) was incubated in 1.1 mL of 50 mM Tris-HCl buffer (pH 8.0) containing nuclease P1 (1 unit), alkaline phosphatase (12 units), and 10 mM $MgCl_2$ at 37 °C for 4 h. Aliquots of these solutions were analyzed by RP-HPLC. The chromatogram of the digested d(T_6DT_5) showed the peaks of the diazoate and 2′-deoxythymidine (dThd), whereas that of d(T_6UT_5) showed dUrd and dThd.

Quantitation Procedures. The concentrations of products were estimated from integrated peak areas of HPLC chromatograms and molar extinction coefficients at 260 nm (ϵ_{260nm}). The ϵ_{260nm} of the diazoate was estimated as 7.3×10^3 M^{-1} cm^{-1} from integration of the H5 and H6 proton signals and RP-HPLC peak area detected at 260 nm relative to those of dCyd. The values of 7.4×10^3 and 1.01×10^4 M^{-1} cm^{-1} were used for dCyd and dUrd, respectively. In the quantitative analysis of the reaction products, the initial RP-HPLC peak area was used as a standard. The values of ϵ_{260nm} for oligodeoxynucleotides were calculated by the nearest neighbor approximation (*26*) and were 1.10×10^5 M^{-1} cm^{-1} for d(T_6CT_5), 1.13×10^5 M^{-1} cm^{-1} for d(T_6UT_5), and 1.80×10^5 M^{-1} cm^{-1} for d(A_5GA_6). The value of 1.10×10^5 M^{-1} cm^{-1} for d(T_6DT_5) was calculated by the previously reported procedure (*10*, *27*).

Melting Curves. Absorbance vs temperature melting curves were measured at 260 nm on a Shimadzu UV-260 UV−vis spectrophotometer equipped with an SPR-5 temperature controller. The total strand concentration of the sample was 10 μM in 100 mM sodium phosphate buffer (pH 7.4). The oligodeoxynucleotide duplexes were premelted at 60 °C and subsequently annealed by reducing the temperature to 4 °C at the rate of 1 °C/min. UV melting curves were obtained by measuring absorbance at 260 nm with a temperature increase from 4 to 50 °C at the rate of 0.5 °C/min.

RESULTS

Detection and Identification of the Intermediate. When 10 mM dCyd was incubated with 100 mM $NaNO_2$ in 1.0 M acetate buffer (pH 3.7) for 5 min at 37 °C, three major peaks appeared in addition to void peaks (t_R = 2.1 and 2.2 min) containing nitrite and nitrate ions in the RP-HPLC chromatogram (Figure 1). The first peak (t_R = 6.9 min) was due to unreacted dCyd. The second peak (t_R = 8.6 min) was attributed to dUrd, a deamination product of dCyd. The retention time and the UV spectrum detected on line for the peak were consistent with those for the authentic dUrd. An unknown product (referred to as **1**) was eluted as the third peak (t_R = 10.6 min), which showed a UV spectrum with λ_{max} = 285 and 310 nm (Figure 1, inset). **1** was isolated by preparative RP-HPLC and subjected to structural assignment. The ^1H NMR spectrum of **1** (in DMSO-d_6 at 22 °C) showed two aromatic protons, H5 and H6, largely shifted to the downfield relative to those of dCyd (Figure 2). In addition, a set of signals of the intact 2′-deoxyribose moiety was observed. No exchangeable proton signal expected for an amino or imino group was observed. **1** was trapped completely by an anion-exchange resin (Dowex 1-X8, OH^- form)

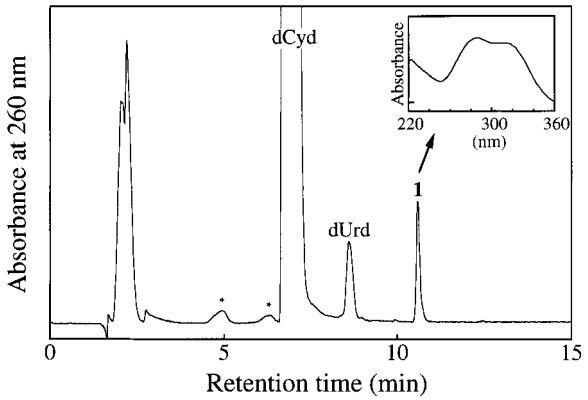

FIGURE 1: RP-HPLC chromatogram for HNO_2-treated dCyd. An HPLC system equipped with an ODS column (4.6 × 150 mm) was used. The eluent was 100 mM triethylammonium acetate buffer (pH 7.0) containing CH_3CN and the flow rate was 1.0 mL/min. The CH_3CN concentration was increased from 0 to 15% for 15 min in the linear gradient mode. Inset is an on-line detected UV spectrum of **1**. The peaks indicated by asterisks are impurities present in starting dCyd.

but passed without retainment through the same bed volume of a cation-exchange resin (Dowex 50W-X2, H^+ form). Negative ion APCI (atmospheric pressure chemical ionization) LC/MS using 100% CH_3CN as an eluent showed signals with *m*/*z* 260, 255, and 227 for **1** (Figure 3). The signals of *m*/*z* 260 and 227 were also observed for the authentic dUrd (MW = 228) with the similar relative intensity. In general, alcohols show a signal originated by the addition of O_2 and H in the negative ion APCI LC mass spectrum (*28*, *29*). The signal with *m*/*z* 260 is likely to be derived from the addition of O_2 and H to the *m*/*z* 227 fragment. Aromatic diazoate compounds (R−NNO^-) are known to give rise to a strong signal of the [M^- − 28] fragment that is produced by the loss of a molecular nitrogen through four−center skeletal rearrangement (*30*, *31*). Thus, the peak with *m*/*z* 255 was assigned as the intact ion (M^-) of **1** containing a diazoate group. The signal with *m*/*z* 227 was attributed to the corresponding $-N_2$ product (R−O^-), i.e., a negative ion of dUrd. Combining these data, we have concluded that **1** is a diazoate derivative of dCyd, 1-(β-D-2′-deoxyribofuranosyl)-2-oxopyrimidine-4-diazoate (Figure 3, inset). It is not clear so far whether the diazoate has Z (syn) or E (anti) form for the N−N double bond.

Reaction Kinetics for HNO_2 Treatment. Figure 4A shows the time courses of concentration changes in the diazoate and dUrd when dCyd was treated with HNO_2 at pH 3.7. The concentration of the diazoate increased rapidly in the early stage of the reaction and reached a plateau, showing a profile characteristic of a reaction intermediate. The maximum concentration of the diazoate was 37 μM at 25 min, and the steady-state concentration (the concentration at 60 min) was 35 μM. The concentration of the diazoate was greater than that of dUrd up to 10 min of the reaction. The time courses at pH 4.7 are shown in Figure 4B. Although concentrations of the diazoate and dUrd were lower than those at pH 3.7, the concentration of the diazoate was greater than that of dUrd up to 50 min.

To assess the influences of thiocyanate, a catalyst of nitrosation (*32*, *33*), and ascorbate, an inhibitor of nitrosation (*34*, *35*), on the formation of the diazoate, dCyd was treated by HNO_2 at pH 3.7 in the presence of 500 mM NaSCN or

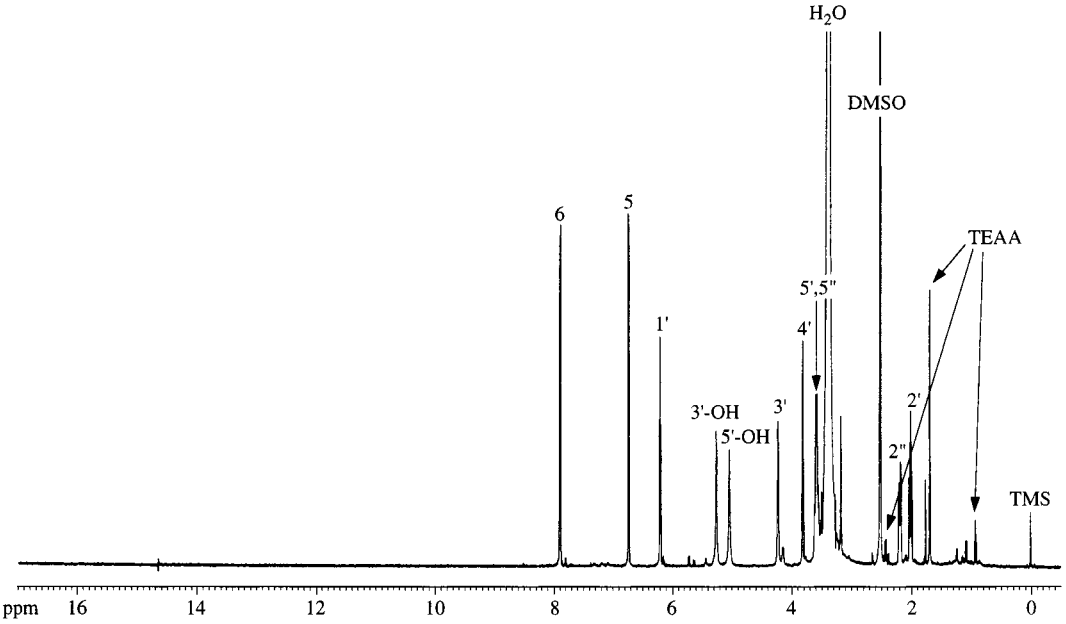

FIGURE 2: ^{1}H NMR spectrum of **1** in DMSO-d_6. The spectrum was measured using an ARX-500 spectrometer (500 MHz) at 22 °C. The signal assignments were performed by DQF-COSY, ^{1}H-^{13}C HMQC, and ROESY (200 ms). The numbers over the resonances designate the atomic position of **1** (see also Figure 3, inset). Extra peaks denoted as TEAA were due to the residual triethylammonium acetate buffer used in the purification process.

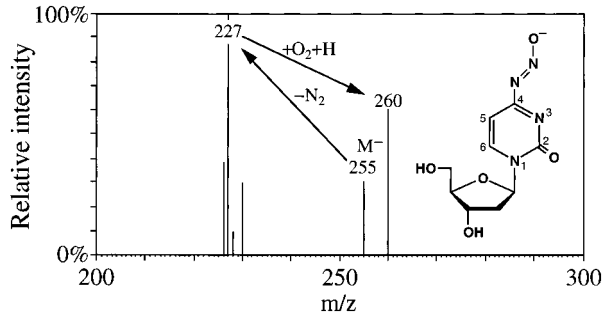

FIGURE 3: Negative-ion APCI-LC mass spectrum of **1**. An MS system equipped with an HPLC system without column was used. The MS conditions: eluent, 100% CH_3CN (isocratic); flow rate, 1 mL/min; vaporization temperature, 300 °C; desolvation temperature, 305 °C; drift voltage, −195 V. The inset shows the structure of **1**, a diazoate derivative of dCyd [1-(β-D-2′-deoxyribofuranosyl)-2-oxopyrimidine-4-diazoate].

100 mM ascorbic acid. When the reaction was performed with NaSCN, the maximum concentration of the diazoate increased up to 88 μM at 4 min and the steady-state concentration was 62 μM (after 50 min) (Figure 4C). In contrast, addition of ascorbic acid reduced the concentration of the diazoate and the maximum concentration was 12 μM at 55 min (Figure 4D).

Formation of the Diazoate by NO. dCyd (10 mM) was dissolved in 10 mL of 100 mM phosphate buffer (pH 7.4) at 37 °C in an open vessel, and NO (99.8%), which was run through a soda lime, was bubbled at the flow rate of 1.0 mL/s through a glass frit into the well stirring solution under aerobic conditions. The pH of the solution was maintained between 7.2 and 7.6 by the titration of 1 N NaOH throughout the reaction. Otherwise, the pH of the solution decreases due to nitrite and nitrate formed by autoxidation of absorbed NO. The RP-HPLC analysis revealed that the diazoate was also generated in addition to dUrd. The yields of the diazoate and dUrd are plotted against the mole of the absorbed NO (measured as the mole of NaOH required to neutralize the solution) in Figure 5. Similar to the HNO_2 treatment (Figure 4), the concentration change in the diazoate showed a profile characteristic of a reaction intermediate. The concentration of the diazoate was greater than that of dUrd up to 15 mmol of NO absorption.

Stability of the Diazoate Intermediate. To evaluate the stability of the diazoate, the purified diazoate (0.7 mM) was incubated under acidic (pH 3.7) and neutral (pH 7.4) conditions and the reaction was monitored by RP-HPLC. At pH 3.7 (1.0 M acetate buffer, 37 °C), the diazoate disappeared with the first-order rate constant 4.8×10^{-4} s^{-1} ($t_{1/2}$ = 24 min) (Figure 6A). On the other hand, at pH 7.4 (100 mM phosphate buffer, 37 °C), the diazoate was much more stable and hydrolyzed very slowly with the rate constant of 5.8×10^{-7} s^{-1} ($t_{1/2}$ = 330 h) (Figure 6B). Both at pH 3.7 and 7.4, the diazoate gave rise to dUrd exclusively. There was no indication of products other than dUrd in the RP-HPLC analysis.

The Diazoate Is Not a Substrate for Uracil-DNA Glycosylase! To elucidate whether the diazoate generated in DNA was removed by uracil-DNA glycosylase, the diazoate containing oligodeoxynucleotide, d(T_6DT_5) (*D* = the diazoate), and d(T_6UT_5) (U = Ura) were prepared from d(T_6CT_5) by HNO_2 treatment and subsequent separation as described in the Materials and Methods. d(T_6DT_5) or d(T_6UT_5) (0.8 μM) was incubated with 0.1 units/mL of uracil-DNA glycosylase at 37 °C in 63 mM Tris-HCl (pH 8.0). The peak of d(T_6UT_5) (t_R = 17.1 min, λ_{max} = 270 nm) in the RP-HPLC chromatogram was completely converted to another peak (t_R = 12.9 min, λ_{max} = 270 nm) after 10 min of incubation (data not shown). Since the product in the new peak decomposed readily by n-butylamine treatment, it was assigned as d(T_6abT_5) (ab = abasic site). On the other hand, d(T_6DT_5) remained intact even at the incubation time of 120 min, and no product was detected by RP-HPLC.

Duplex Stability of Oligodeoxynucleotide Containing the Diazoate. To elucidate the thermodynamic effect of the

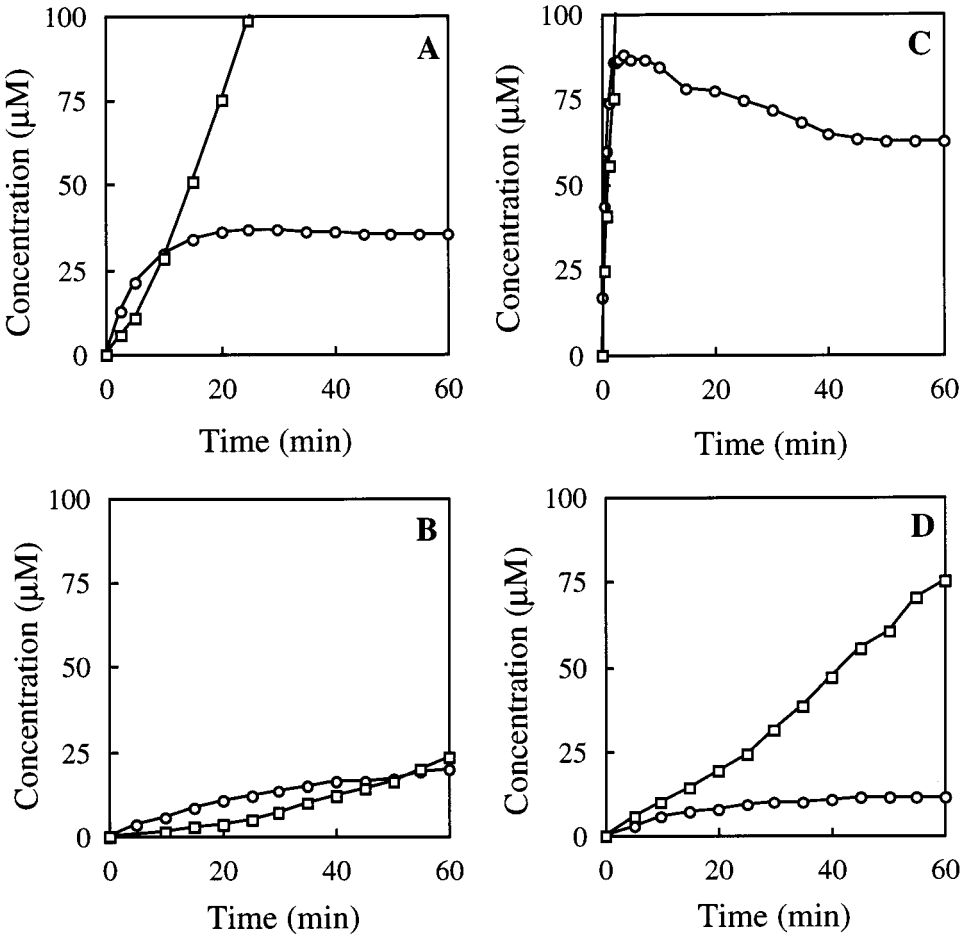

FIGURE 4: Formation of the diazoate and dUrd by HNO_2 treatment when 10 mM dCyd was incubated at 37 °C with 100 mM $NaNO_2$ in 1.0 M acetate buffer at (A) pH 3.7 and (B) pH 4.7. Effects of (C) thiocyanate and (D) ascorbate on the formation of the diazoate and dUrd. The reaction conditions were the same as in panel A (pH 3.7) except that 500 mM NaSCN or 100 mM ascorbic acid was added. The concentrations of the diazoate (circle) and dUrd (square) were plotted against the reaction time. Products were analyzed by RP-HPLC.

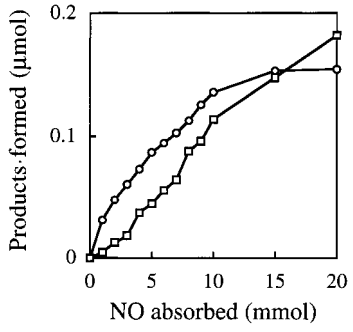

FIGURE 5: Correlation of the amounts of the diazoate (circle) and dUrd (square) with the amount of absorbed NO. 10 mM dCyd was dissolved in 10 mL of 100 mM phosphate buffer (pH 7.4) at 37 °C in an open vessel, and NO was bubbled at the flow rate of 1.0 mL/s through a glass frit into the well stirring solution under aerobic conditions. The pH was held in the range 7.2−7.6 by the titration of 1 N NaOH. The amount of NO absorbed means the amount of NaOH added.

diazoate lesion on the duplex stability, UV-melting measurements were performed for three duplexes, $d(T_6DT_5)\cdot d(A_5GA_6)$, $d(T_6UT_5)\cdot d(A_5GA_6)$, and $d(T_6CT_5)\cdot d(A_5GA_6)$, which contain D:G, U:G, and C:G pairs at the same position, respectively. All these duplexes showed a single transition in the UV melting curves (Figure 7). The T_m values were 21.2 °C for $d(T_6DT_5)\cdot d(A_5GA_6)$, 18.2 °C for $d(T_6UT_5)\cdot d(A_5GA_6)$, and 30.1 °C for $d(T_6CT_5)\cdot d(A_5GA_6)$. The T_m value for the diazoate duplex as well as the Ura duplex was significantly low in comparison with that for the correctly paired Cyt duplex.

DISCUSSION

HNO_2 induces G·C → A·T transitions most frequently (44−56% of total mutations) (*36*). The predominant mutations resulting from NO treatment by different NO donors are also G·C → A·T transitions (*14*, *25*, *37*), although there is an exception where NO bubbling resulted in A·T → G·C transitions (61−75%) as well as G·C → A·T transitions (23−29%) (*38*). The G·C → A·T transition is potentially important because it has been observed in numerous human diseases (*39*, *40*). This transition could arise from deamination of either Gua to Xan or Cyt to Ura. The contribution of Xan and Ura to the transition could be roughly estimated by the formation rate and the frequency of miscording during DNA replication. In HNO_2 treatment of thymus DNA, the rate of formation of Xan is 2.2-fold higher than Ura (*5*). In the reaction with NO, the formation rate of Xan is reported to be 1.7−2.0-fold higher than that of Ura for not only nucleosides but also single- and double-stranded oligodeoxynucleotides (*41*). In the both studies, the nucleobases were quantified after acid treatment of the samples. Therefore, it is very likely that the acid-labile diazoate, even if present, would have been completely converted to Ura. Xan in oligodeoxynucleotide templates directs incorporation of the

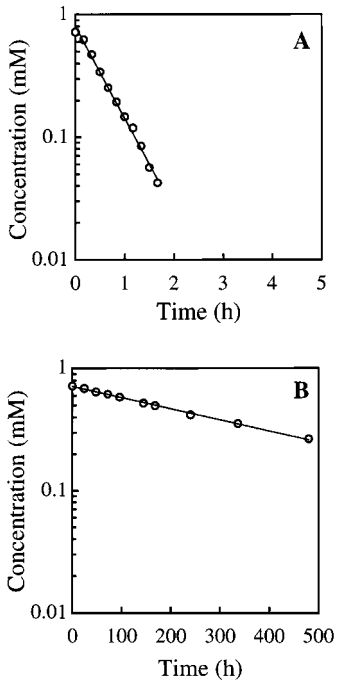

FIGURE 6: Exponential plots of the diazoate concentration vs the incubation time. The purified diazoate (0.7 mM) was incubated at 37 °C and (A) pH 3.7 (1.0 M acetate buffer) and (B) pH 7.4 (100 mM phosphate buffer), and the concentration was determined by RP-HPLC.

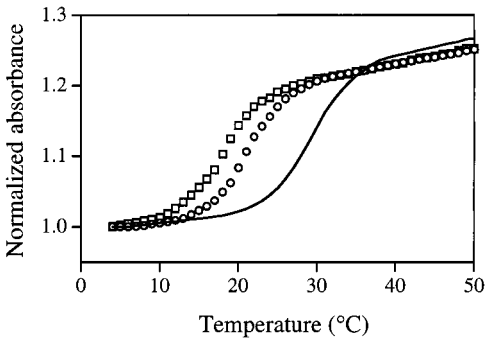

FIGURE 7: Normalized UV thermal denaturation profiles for the duplexes, d(T_6DT_5)·d(A_5GA_6) (circle), d(T_6UT_5)·d(A_5GA_6) (square) and d(T_6CT_5)·d(A_5GA_6) (solid line). The total strand concentration of each sample was 10 μM in 100 mM sodium phosphate buffer (pH 7.4). Melting profiles were obtained by measuring UV absorbance at 260 nm. The temperature was raised at the rate of 0.5 °C/min for the measurement.

nucleotides with the ratio of T:C:A:G = 100:60:17:14 when the reaction was catalyzed by Drosophila Polymerase α in vitro (*42*). This suggests that the probability of G·C → A·T transition mutation is 52% [= 100/(100 + 60 + 17 + 14)] when DNA replication passes a single Xan site. On the other hand, Ura in DNA codes exactly like Thy and generates G·C → A·T transition quantitatively (*43*, *44*). Considering the relative yield and the miscording frequency of Xan and Ura mentioned above, the contribution of Ura derived from Cyt to HNO_2- or NO-induced G·C → A·T transitions would be approximately one-half of the total G·C → A·T transitions. In living cells, Ura is continuously formed from Cyt by spontaneous hydrolysis (*45*, *46*). Thus, a repair enzyme for this DNA damage, uracil-DNA glycosylase, exists abundantly in prokaryotic and eukaryotic organisms (*47*). The importance of this enzyme in mutagenesis has been confirmed by the fact that *E. coli* deficient in uracil-DNA glycosylase exhibits a 30-fold increase in G·C → A·T transition mutations (*48*). Accordingly, Ura produced by HNO_2 or NO in DNA should also be excised by uracil-DNA glycosylase. Recently, Hartman et al. examined the mutational effect of HNO_2 to *E. coli* employing several DNA repair deficient mutants (*23*). An ung$^-$ strain deficient in uracil-DNA glycosylase displayed no phenotype to cell killing, and little increase (less than 2-fold) was observed in the mutation frequency (rifanpicine resistance) relative to an ung$^+$ strain. Conversely, the mutation frequency increased ca. 10-fold with a strain deficient in Uvr nuclease (*49*) which excises pyrimidine dimer and various bulky adducts. Schmutte et al. treated a double-stranded plasmid in vitro with NO gas or spermine/nitric oxide complex (SPER/NO, an NO donor) at pH 7.4 and transformed it into ung$^-$ and ung$^+$ strains (*24*). With the ung$^-$ strain, no significant increase in the reversion frequency over the ung$^+$ strain was observed. They also treated the ung$^-$ and ung$^+$ strains with SPER/NO in vivo after transformation by the intact plasmid. The in vivo exposure resulted in comparable reversion frequencies for ung$^-$ and ung$^+$ strains. On the basis of these results, the authors concluded that the mutagenicity of NO was not caused by deamination of Cyt in double-stranded DNA in *E coli*. To obtain more precise information, Merchant et al. performed a similar study on the NO-induced mutation in the single-stranded region of DNA (*25*). A gapped plasmid was treated with diethylamine/nitric oxide complex (DEA/NO, an NO donor) at pH 7.4 and transformed it into ung$^-$ and ung$^+$ strains. The NO treatment caused only a 2-fold increase in the reversion frequency for ung$^-$ strain. These three studies employing the ung$^-$ strain demonstrate that the mutation via the formation of Ura in DNA is not a dominant mechanism of G·C → A·T transitions caused by HNO_2 and NO treatments.

In the present study, we have shown that the diazoate derivative of dCyd is formed as an intermediate in the reaction of dCyd with HNO_2 or NO. The maximum yield was 0.37% relative to starting dCyd in the reaction with HNO_2 and 0.15% with NO (without catalyst). The concentration of the diazoate increased rapidly in the early stage of the reaction and reached a plateau, showing a profile characteristic of a reaction intermediate (Figures 4 and 5). In the successive reactions (dCyd → the diazoate → dUrd), the diazoate was the dominant species over dUrd in the initial stage of the reaction. In general, the studies on the mutagenicity of HNO_2 and NO to living cells are performed under mild conditions. For instance, Hartman et al. performed the nitrous acid treatment at pH 4.7 and 37 °C up to 30 min against *E. coli* ung$^-$ and ung$^+$ strains (*23*). In the present study, dCyd was treated by HNO_2 in vitro at the same pH and temperature. Under these conditions, the yield of the diazoate was 2-fold greater than dUrd at the incubation time of 30 min (Figure 4B). The pH of cytoplasm in cells is usually higher than 4.7 used in this work to study the formation of the diazoate (*50*). Under such conditions, the yield of the diazoate relative to dUrd will be higher since the diazoate is more stable at higher pH (Figure 6). Schmutte et al. carried out in vivo NO treatment at pH 7.4 and 37 °C using the SPER/NO concentrations up to 1 mM (*24*), which roughly corresponds to 0.01 mmol of absorbed NO in the present work. Since the ratio of the formed diazoate vs dUrd was 7:1 with 1 mmol of NO absorption (Figure 5), the vast

majority of the reaction product should be the diazoate at the level of 0.01 mmol NO absorption. Thus, the major existing species in the nitrosation reaction of dCyd by HNO_2 or NO is likely to be the dCyd diazoate but not dUrd under in vivo assay conditions.

An oligodeoxynucleotide containing a single diazoate residue, $d(T_6DT_5)$, was obtained from $d(T_6CT_5)$ by HNO_2 treatment, indicating that the diazoate is also formed in oligodeoxynucleotides. The diazoate in $d(T_6DT_5)$ was not a substrate of uracil-DNA glycosylase. This result clearly demonstrates that uracil-DNA glycosylase existed in cells cannot repair the Cyt diazoate formed in DNA. Moreover, it is also consistent with the results that deficiency in uracil-DNA glycosylase has little influence on mutation when *E. coli* cells are treated by HNO_2 or NO (*23*−*25*). The dCyd diazoate was stable under physiological conditions with a half-life of ca. 14 days (Figure 6B), which is long enough for many rounds of DNA replication before the diazoate is converted to dUrd. The T_m value of a duplex containing the diazoate, $d(T_6DT_5)\cdot d(A_5GA_6)$, was much lower than that of a duplex containing a correct base pair, $d(T_6CT_5)\cdot d(A_5GA_6)$. This result indicates that the Cyt diazoate cannot form a stable base pair with correct Gua and suggests that the formation of this lesion in DNA is potentially cytotoxic and/or genotoxic. It is also important to clarify whether the Cyt diazoate is processed by DNA glycosylases other than uracil-DNA glycosylase or nucleotide excision systems.

In conclusion, we have found a previously unidentified product in the reactions of dCyd with HNO_2 and NO, and identified it as a diazoate intermediate. This damage does not form a stable base pair with Gua and is not recognized by uracil-DNA glycosylase. The present results may have significant importance in elucidating genotoxic effects of HNO_2 and NO.

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