2‘-Deoxycytidine Glycols, a Missing Link in the Free Radical-mediated Oxidation of DNA*

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2‘-Deoxycytidine glycols (5,6-dihydroxy-5,6-dihydro-2‘-deoxyctidine) are major products of the hydroxyl radical-induced oxidation of 2‘-deoxycytidine resulting from either a Fenton reaction or exposure to ionizing radiation. Because of their instability, however, the glycols have not previously been characterized. Instead, the impetus has been placed on the primary decomposition products of 2‘-deoxycytidine glycols, which includes 5-hydroxy-2‘-deoxyctidine, 5-hydroxy-2‘-deoxyuridine, and 2‘-deoxyuridine glycols. Here, we have identified one of the four possible diastereomers of 2‘-deoxycytidine glycols by product analyses of decomposition products, 1H NMR, and mass spectrometry. This glycol was observed to decompose with a half-life of 50 min at 37 °C in buffered neutral solutions and preferentially undergo dehydration to 5-hydroxy-2‘-deoxycytidine. The rate of decomposition was strongly dependent on pH (2–10) and the concentration of phosphate ion (10–300 mM). Next, we report on the deamination of cytosine glycols to uracil glycols in oxidized DNA using acid hydrolysis and high performance liquid chromatography analysis with electrochemical detection to monitor 5-hydroxycytosine and 5-hydroxycytosine. The results showed that the lifetime of cytosine glycols is greatly enhanced in DNA (34-fold; half-life, 28 h), and that deamination accounts for at least one-third of the total decomposition. The relatively long lifetime of cytosine glycols in DNA suggests that this important class of DNA oxidative products will be significantly involved in repair and mutagenesis processes.

The main species responsible for oxidative DNA damage in cells appear to be hydroxyl radicals that are generated by a Fenton reaction involving the reduction of H2O2 by transition state metal ions (e.g. Fe2+ or Cu+) (1). In addition, the indirect effects of ionizing radiation may be attributed predominantly to the reaction of hydroxyl radicals with DNA (2). Hydroxyl radical-induced oxidation of DNA is complex, leading to a multitude of modifications at the level of DNA bases (thymine, cytosine, adenine, guanine, and 5-methylcytosine) and the sugar moiety (3, 4). Using simple model systems, a large number of modified nucleosides has been identified (1–6). H2O2-iron-mediated decomposition of 2‘-deoxycytidine (dCyd)1 was shown to lead to the formation of several stable products including oxidative modifications of the base and nucleoside in the product mixture (7). Recently, we identified 17 stable oxidative nucleoside modifications from γ irradiation and menadione photosensitization of dCyd in aerated aqueous solutions (8). The major products of these reactions included glycol, hydrocinnamonic acid, and formamide derivatives, with an intact sugar moiety, similar to the oxidation of 2‘-deoxyuridine (dUrd) and thymidine. In addition, several unique products of dCyd were identified. Although dCyd glycols were not detected in the reaction mixture, the formation of these labile products is inferred by the presence of 5-hydroxy-2‘-deoxycytidine (oh5dCyd) and 2‘-deoxyuridine (dUrd) glycols. It is thought that oh5dCyd and dUrd glycols arise from dehydration and deamination of dCyd glycols, respectively (Scheme 1). In addition, 5-hydroxy-2‘-deoxyuridine (oh5dUrd) may be produced from dehydration of dUrd glycols, although this is a minor pathway for the 2‘-deoxyribonucleosides. The combined yield of oh5dCyd and dUrd glycols in the OH radical induced decomposition of dCyd represents about 40% of the product mixture, suggesting that dCyd glycols are major products of dCyd oxidation.

Oxidative DNA damage to cytosine in DNA is important because it appears to be responsible for C to T transitions, which are the most common type of base substitution in the spectra of bacterial and mammalian genes (9). Recently, there has been much interest in the stable decomposition products of cytosine glycols in DNA, including 5-hydroxycytosine, 5-hydroxycytosine, and uracil glycols. These products are generated in DNA by various free radical and oxidant mediated processes (10). Furthermore, 5-hydroxycytosine appears to be present in cellular DNA of untreated rodent tissues and human lymphocytes at levels that are comparable with those of 8-oxo-7,8-dihydroguanine, as measured by HPLC with electrochemical detection (10, 11). The above oxidative lesions of cytosine have proven to be substrates for several key enzymes of base excision repair including Escherichia coli endonuclease III, endonuclease VIII, formamidopyrimidine N-glycosylase (5-hydroxycytosine), and uracil N-glycosylase (5-hydroxyuracil) (12–16). Initially, 5-hydroxycytosine was reported to be potentially mutagenic, miscoding with either A or C during DNA polymerization with synthetic oligonucleotides and Klenow fragment (17). Subsequently, Loeb and co-workers (18) reported on an oxidative modification of dCTP which, when incorporated into DNA in vitro and then transferred into E. coli, resulted in an unusually high frequency of C to T transitions. The modification was identified as a derivative of 5-hydroxycytosine. However, Essigmann and co-workers (19) recently reported that 5-hydroxycytosine was relatively weakly mutagenic (0.05%).

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2‘-deoxyuridine; dCyd glycols, 5,6-dihydroxy-5,6-dihydro-2‘-deoxycytidine; dUrd glycols, 5,6-dihydroxy-5,6-dihydro-2‘-deoxyuridine; HPLC, high performance liquid chromatography; MS, mass spectroscopy.

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‡ The abbreviations used are: dCyd, 2‘-deoxycytidine; dUrd, 2‘-deoxyuridine; oh5dCyd, 5-hydroxy-2‘-deoxycytidine; oh5dUrd, 5-hydroxy-
Characterization of 2′-Deoxycytidine Glycols

The final step in the synthesis of dCyd glycols involved the hydrolysis of our bromohydron (trans-5(R,6R))-5′-cytidine bromohydron; above). This was accomplished by addition of Ag₂O to an aqueous solution of the bromohydron at 4 °C until formation of a black precipitate. The desired product was purified from the reaction mixture by HPLC using 10 mM ammonium formate (pH 5.5) as the mobile phase. The product (4.6 min) was collected, immediately frozen, and then lyophilized to dryness.

1H NMR analysis (500 MHz, 99.99% D₂O, reference to 3-(trimethylsilyl)-propionate-2,3,3-d₃) 6.602 (spt, 1, H₁), 7.0 (5.5 Hz), 5.2 (d, 1, H₂), 4.7 (d, 1, H₃), 3.6 (dd, 1, H₄), 4.30 (m, 1, H₃), 3.71 (m, 1, H₄), 3.7 (dd, 1, H₅), 2.18 (m, 1, H₂), 2.12 (m, 1, H₃). Electrospray ionization-MS (positive mode) m/z 262 (MH⁺), 244 (MH⁺ - H₂O), 146 (MH⁺ - deoxyribose), 117 (MH⁺ - base).

Oxidation of DNA and Analysis of Oxidized Cytosine Products—To prepare DNA containing cytosine glycols, calf thymus DNA (0.5 mg/ml) was treated with Fe₂⁺ (25 μM), ascorbate (100 μM), and H₂O₂ (0.5 mM) for 1 h on ice. Subsequently, DNA was precipitated twice in the presence of 0.2 mM desferoxamine and dialyzed for 16 h at 4 °C against 10 mM phosphate buffer (pH 7.0) and 0.1 mM EDTA. The levels of 5-hydroxycytosine and 5-hydroxyuracil were determined in oxidized DNA by HPLC coupled to electrochemical detection. Practically, DNA (100 μg) was precipitated and resuspended in 88% formic acid (400 μl) and heated to 140 °C for 45 min, and the mixture was taken to dryness under reduced vacuum. The residue was first dissolved in 500 μl of the HPLC solvent buffer, consisting of 50 mM phosphate buffer (pH 5.5) and 0.1% methanol at 70 °C in a 2 ml vial. The mixture was then injected into the HPLC. The separation of 5-hydroxycytosine and 5-hydroxyuracil was achieved by using a 5-μm AQ C-18 column (inner diameter, 250 × 4.6 mm) from YMC (Wilmington, NC). Elution of compounds was achieved at a flow rate of 1.4 ml/min with 50 mM phosphate (pH 5.5) and 0.1% methanol as the mobile phase. Under these conditions, the retention times of 5-hydroxycytosine and 5-hydroxyuracil were 6.5 and 8.0 min, respectively. Cytosine eluting at 7.0 min did not interfere with the detection of modified bases. The electrochemical cell consisted of a dual electrode flow cell (model 5011, ESA Inc.) was set at 175 and 425 mV to optimize the detection of 5-hydroxycytosine and 5-hydroxyuracil, respectively. The column was washed between analyses with 20% methanol in 50 mM phosphate buffer (pH 5.5) in order to eliminate slowly eluting DNA components from the column. The levels of 5-hydroxycytosine and 5-hydroxyuracil were normalized for the amount of DNA injected on the basis of the UV signal for cytosine recorded on the same chromatographic run. All compounds were calibrated with commercially available or synthetic standards (22).

RESULTS

A new product was purified from the hydrolysis of dCyd bromohydrons. On the basis of chemical studies and spectroscopic measurements, this product was unambiguously identified as a diastereomer of dCyd glycol. Initial studies with HPLC indicated that the product transformed into the cis-dCyd and, to a minor extent, into a cis-dUrd glycol, at 37 °C and pH 7 (Fig. 1). The formation of these stable products can only be explained by the decomposition of an intermediate dCyd glycol. Subsequently, we investigated the structure of this intermediate by 1H NMR and MS analyses. The 1H NMR spectrum of dCyd glycol was similar to that of dUrd and 5-methyl-2′-deoxycytidine glycols (8, 23). The spectrum exhibited two proton signals at 4.7 and 5.2 ppm, which may be assigned to the H(5) and H(6) protons of the pyrimidine base, respectively. In addition, there were several protons that may be assigned to the 2-deoxyribose moiety (H(2′), H(2′), H(3′), and H(4′)), although it was necessary to examine the sample by two-dimensional NMR in order to discern these protons from those of cis-dCyd, a minor contaminant in these analyses (results not shown). Additional confirmation of the structure was obtained by MS analysis.

EXPERIMENTAL PROCEDURES

Chemicals were purchased from either Sigma or Aldrich with the highest available purity. The HPLC system was composed of a dual piston pump (model 510, Waters Associates, Milford, MA) equipped with either a manual injector (model 7125, Rheodyne, Berkeley, CA) or automatic injector (model 710B, Waters Associates), a UV detector (model 7125, Waters Associates), a manual injector (model 710B, Waters Associates), and an electrochemical detector (model 5200A, ESA Inc., Chelmsford, MA). Data were collected by means of an AD converter (SAT/RIN, Waters Associates) connected to data acquisition software (Millennium 2010, Waters Associates). 1H NMR analyses were performed in Fourier transform mode using a Varian U500 instrument (Palo Alto, CA). Mass analysis was carried out on a Perkin-Elmer Scieix API III triple quadrupole apparatus equipped with a nebulizer-assisted electrospray ionization source. Oh-2′Cyd and oh-2′Urd and the corresponding bases were prepared by established methods (21, 22).

Synthesis of 5,6-Dihydroxy-5,6-dihydro-2′-deoxycytidine (dCyd Glycols)—The synthesis of dCyd glycols was accomplished in two steps via the decomposition of an intermediate dCyd glycol. Subsequently, we investigated the structure of this intermediate by 1H NMR and MS analyses. The 1H NMR spectrum of dCyd glycol was similar to that of dUrd and 5-methyl-2′-deoxycytidine glycols (8, 23). The spectrum exhibited two proton signals at 4.7 and 5.2 ppm, which may be assigned to the H(5) and H(6) protons of the pyrimidine base, respectively. In addition, there were several protons that may be assigned to the 2-deoxyribose moiety (H(2′), H(2′), H(3′), and H(4′)), although it was necessary to examine the sample by two-dimensional NMR in order to discern these protons from those of oh-dCyd, a minor contaminant in these analyses (results not shown). Additional confirmation of the structure was obtained by MS analysis.
These analyses clearly depicted the molecular ion of dCyd glycols (262 m/z) as well as a major fragment at 244 m/z, indicating the loss of water from the molecular ion. The loss of water is a characteristic feature observed in MS spectra of thymidine and 2'-deoxyuridine glycols (8, 23).

2'-Deoxycytidine glycols exist as four possible diastereomers, depending on the configuration of the substituents at C(5) and C(6) of the 5,6-saturated pyrimidine ring. Assignment of the configuration for the dCyd glycol under study (cis-(5S,6R)) is consistent with the mechanism of hydrolysis of pyrimidine bromohydrins based on previous studies with thymine, thymidine, and 2'-deoxyuridine bromohydrins (Refs. 5, 8, and 24–26 and Scheme 2). The first step of the mechanism involves an anisotropic rearrangement in which the vicinal hydroxyl group at C(6) of the bromohydrin displaces the bromide ion at C(5). This step leads to the formation of an epoxide (I) and/or zwitterion (II) intermediate (Scheme 2). Subsequently, the addition of water to the epoxide gives a trans-glycol, whereas addition to the zwitterion gives either a trans- or cis-glycol depending on which side that water attacks the zwitterion. These reactions are complicated by the fact that trans-glycol undergoes epimerization at C(6) to give the corresponding cis-glycol. Nevertheless, products with a cis-configuration at C(6) are preferentially formed in the reaction of water as well as other nucleophiles such as H$_2$O$_2$ with pyrimidine bromohydrins so far studied (5, 8, 24–26). This suggests that a zwitterion is probably the main intermediate of bromohydrin hydrolysis and that the subsequent addition of water is directed to the same side of the molecule as the hydroxyl group at C(5) because of polar interactions with this group. Thus, in conclusion, the first step in the hydrolysis of pyrimidine bromohydrins causes inversion of the configuration at C(5), whereas the second step leads to two diastereomers that is, the one with retention of the configuration at C(6), i.e. the cis-diastereomer, is the major product. Taking the latter points into account, the hydrolysis of trans-(5R,6R) dCyd bromohydrin should give a glycol with a cis-(5S,6R) configuration as the major product. Interestingly, we also separated a second dCyd glycol in minor quantities from the hydrolysis mixture. According to the mechanism of hydrolysis, this glycol may be tentatively assigned as a trans-(5S,6S) dCyd glycol. Also, this is consistent with the fact that the glycol eluted before the corresponding cis-glycol, i.e. cis-(5S,6R) dCyd glycol, on reversed phase chromatography and that it appeared to isomerize into a cis-glycol upon standing at room temperature.

The chemical properties of the minor dCyd glycol are very similar to those of the trans-diastereomers of dUrd and thymidine glycols (24, 27, 28).

Finally, the proposed stereochemistry of cis-(5S,6R) dCyd glycol is supported by $^1$H NMR analyses that depicted a clear difference between cis- and trans-diastereomers. The C(6) hydroxyl group of pyrimidine glycols exerts an influence on the conformational features of the sugar ring, which may be described in terms of a dynamic pseudorotational cycle between two puckered forms. The state of this equilibrium is reflected by the magnitude of trans-coupling constants. In particular, the coupling constant for H(1') and H(2') of pyrimidine glycols is significantly lower for diastereomers with a 6R configuration (6–7 Hz) compared with those with a 6S configuration (8–9 Hz) (27). Furthermore, the NMR studies of pyrimidine glycols are consistent with x-ray crystallography analyses that have established the absolute configuration of cis-(5R,6S) and cis-(5S,6R) diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine (29, 30). As for the dCyd glycol under study here, the coupling constant of H(1') and H(2') was 7.0 Hz, and thus, the C(6) position of this glycol has an R configuration. Together with the well-established stereochemistry of bromohydrin hydrolysis, the results of NMR analysis indicate that the new compound is a dCyd glycol with a cis-(5S,6R) configuration.

The decomposition of the dCyd glycol was monitored as a function of incubation time at 37 °C by HPLC with UV detection at 214 nm (Fig. 1). The rate of decomposition followed unimolecular kinetics with rates in the range from 0.0075 to 0.070 min$^{-1}$, corresponding to half-lives ranging from 10 to 90 min (Fig. 2). The half-life was strongly dependent on pH (Fig. 3). Over a range of values from pH 3 to pH 10, we observed a maximum rate of decomposition between pH 4 and pH 5. In this range, the amount of oh$^3$dCyd represented approximately 90% of the total decomposition of dCyd glycol. Above pH 10, however, the rate of decomposition rapidly increased, which may be attributed partly to dehydration of the glycol into oh$^3$dCyd and partly to base-induced fragmentation of the base moiety. In addition to the effect of pH, the rate of decomposition of dCyd glycol depended on the concentration of phosphate with an exponential increase going from 5 to 50 mM, followed by a linear increase from 50 to 300 mM (Fig. 4). Again, dehydration of dCyd glycols into oh$^3$dCyd was the predominant process. To assess the decomposition of intermediate cytosine glycols in double-stranded DNA, we incubated freshly oxidized DNA (0.5 mg/ml) at 37 °C in the presence of 10 mM phosphate (pH 7.0) and 0.1 mM EDTA for periods of time ranging from 12 to 200 h. The levels of 5-hydroxycytosine and 5-hydroxyuracil in DNA were measured by acid hydrolysis and HPLC analysis. This treatment leads to the complete release of modified and nonmodified bases from DNA and does not entail the decomposition of either 5-hydroxycytosine or 5-hydroxyuracil (31). Under these conditions, however, uracil glycols transform quantitatively into 5-hydroxyuracil (31–33). In addition, we
observed an analogous transformation for dCyd glycols. When purified dCyd glycols were treated under identical conditions as DNA, the glycols transformed into 5-hydroxycytosine with minor amounts of 5-hydroxyuracil (<10%; results not shown). Thus, for the analyses of DNA, we assume that 5-hydroxycytosine represents the combined total of cytosine glycols and 5-hydroxycytosine, whereas 5-hydroxyuracil represents the combined total of uracil glycols and 5-hydroxyuracil.

The changes of 5-hydroxycytosine to 5-hydroxyuracil observed as a function of incubation of oxidized DNA at pH 7 and 37 °C may be attributed to the deamination of cytosine glycols in DNA. The rate of deamination of cytosine glycols to uracil glycols in oxidized DNA was calculated to be 9.6 × 10^{-3} h^{-1} according to a least squares best fit of our data to an exponential function for the loss of 5-hydroxycytosine (((P_t - P_o)/(P_o - P_s)) versus time) and for the growth of 5-hydroxyuracil (((P_o - P_s)/(P_o - P_t)) versus time), where P is the amount of 5-hydroxycytosine or 5-hydroxyuracil at initial times (P_o), intermediate times (P_i), and infinite times (P_s) of incubation. On the basis of the initial and final amounts of 5-hydroxycytosine (1140 and 700 fmol/nmol cytosine, respectively), the percentage of deamination may be estimated to be at least 38% of the total decomposition (deamination plus dehydration). This may be considered to be a low estimate because we assume that no 5-hydroxycytosine is present in oxidized DNA at the beginning of incubation. The presence of 5-hydroxycytosine in DNA initially would lead to a higher estimated value for the contribution of deamination. Nevertheless, assuming that deamination is 38% of the total decomposition, then the total rate of decomposition including both deamination and dehydration of cytosine glycols in DNA may be estimated to be 2.5 × 10^{-2} h^{-1} (9.6 × 10^{-3} h^{-1} × 1/0.38). This value corresponds to a half-life of 28 h for cytosine glycols in DNA.

**DISCUSSION**

The deamination of cytosine is a potentially mutagenic event in cellular DNA because it generates uracil, which pairs with adenine during DNA synthesis. This gives rise to C to T transition mutations. For example, the spontaneous deamination of cytosine to uracil and 5-methylcytosine to thymine are considered to be a major source of human mutations (34, 35). Further...
thermore, the deamination of cytosine hydrates, cytosine-containing 6–4 pyrimidine-pyrimidone adducts, and particularly cytosine-containing pyrimidine cyclobutyl-dimers have been implicated in solar mutagenesis (36, 37). In contrast, there has been relatively little work devoted to elucidating the pathway of mutagenesis induced by oxidative damage to cytosine despite the fact that cytosine is a preferential site for mutations and that saturation of the 5,6-double bond renders this base highly susceptible to deamination. The chemistry of dCyd glycols ultimately determines the partition between a weakly mutagenic product (oh\textsuperscript{\textregistered}dCyd) that arises from dehydration and a strongly mutagenic product (dUrd glycols) that arises from deamination. As a first step toward understanding this pathway, we have characterized one of the four diastereomers of dCyd glycols.

The chemistry of dCyd glycols is strikingly similar to that of photohydrates (6-hydroxy-5,6-dihydropyrimidines) produced by UV irradiation of cytosine and its derivatives. Johns and co-workers (38, 39) studied these products several years ago. The difference in structure between glycol and photohydrate products is that the latter lacks an OH substituent at C(5). For these products, there are two possible pathways of decomposition, which includes dehydration and deamination. Also, in both cases, dehydration is the preferred pathway by at least a factor of 10. The half-life for the decomposition of dCyd glycols is 50 min at 37 °C, whereas the half-life for dCyd photohydrates under similar conditions is about 29-min (the half-life of dCyd hydrates at 37 °C is calculated from data at 25 °C assuming an energy of activation of 13 kcal/mol; Ref. 38). Furthermore, pH and the concentration of salt affected the decomposition of cytidine photohydrates in a manner similar to that of dCyd glycols. For example, the rate of decomposition reached a maximum at pH 5 for cytidine photohydrates compared with pH 4–5 for our dCyd glycol. Also, an increase in the concentration of salts in both reactions led to an increase in the rate of decomposition. To explain these phenomena, Johns and co-workers (39) proposed that the decomposition of cytidine hydrates involved general base catalysis. Below pH 6, cytidine hydrates become protonated (pK\textsubscript{a} = 5), and thus, the reaction of the conjugate base of the buffer with protonated cytidine hydrates is enhanced. At lower pH (pH 5), however, the rate begins to fall because the conjugate base becomes protonated. In support of this mechanism, the effect of pH was not observed in the absence of salt; however, Johns and co-workers (39) observed an effect for the hydrate of cytidine monophosphate and proposed that this occurs as a result of self-catalysis.

Our results suggest that the lifetime for the decomposition of cytosine glycols in DNA is much longer than that for the monomer and secondly that the ratio of dehydration to deamination is greatly shifted toward deamination in the case of DNA (Fig. 5). It is not surprising that DNA structure affects the decomposition of cytosine glycols because the double helix will change the extent of protonation as well as the interaction of water and conjugate base with the glycol. The effect of DNA helix structure on the stability of cytosine products has been studied for a number of modifications. For example, the rate of deamination for cytosine is about 140-fold slower in double-stranded compared with single-stranded DNA (40). In contrast, the rate of deamination of cytosine containing cyclobutyl pyrimidine dimers does not appear to change when comparing the half-life of cis-syn cyclobutyl dimer of dCpdT with that of cyclobutyl dimers in double-stranded DNA (7.6 h compared with 5.0 h). In fact, the kinetic and thermodynamic parameters as well as the effect of pH over the range pH 2–10 are very similar for the deamination of cyclobutyl dimers in the two systems (41, 42). However, the lack of an effect for cyclobutyl dimers may be due to disruption of DNA structure near the damaged site. In the case of cytosine hydrates, which closely resemble cytosine glycols in structure and chemistry, the lifetime is enhanced by 37-fold when comparing cytosine hydrates in alternating copolymers of poly(dG-dC) (half-life, 18 h) and dCyd hydrates as monomers in solution (half-life, 29 min) under neutral conditions at 37 °C (43). Additional studies of cytosine hydrates in DNA showed that the extent of dehydration (>90%) compared with deamination (<10%) and the effect of pH on deamination were similar for both cases involving either the monomer or the polymer (44). Thus, the latter studies indicate that DNA structure greatly increased the half-life of cytosine hydrates (37-fold), although the partition between dehydration and deamination is not affected. In comparison, the lifetime of cytosine glycols is increased by 34-fold in oxidized calf thymus DNA (half-life, 28 h) compared with dCyd glycols in solution (half-life, 50 min) under neutral conditions at 37 °C. Thus, DNA structure enhances the stability of cytosine glycols and hydrates to a similar extent. On the other hand, the partition between the deamination of cytosine hydrates and deamination of cytosine glycols is shifted toward deamination in DNA (deamination > 38%) compared with dCyd glycol (deamination < 10%). Thus, in the case of cytosine glycols, the double-stranded structure of DNA not only enhances the stability of these products but also affects the mechanism of decomposition into stable products.

In view of the long lifetime of cytosine glycols in DNA, the majority of these modifications will probably be removed from cellular DNA by base excision repair, assuming that cytosine glycols are as good substrates as thymine and uracil glycols for endonuclease III and related enzymes. Moreover, they are sufficiently long-lived to act as either blocking or miscoding lesions during DNA synthesis, similar to the processing of other stable oxidative modifications. Interestingly, Loeb and co-workers (18) observed an unusually high frequency (2.5%) of mutations (C to T transitions) for an oxidation product of dCTP incorporated into DNA in vitro with immunodeficiency virus reverse transcriptase and then transferred into E. coli (this assay was referred to as reverse chemical mutagenesis). The putative product of dCTP oxidation was identified as the 5-hydroxycytosine derivative on the basis of HPLC and mass spectrometry. However, a more recent study indicates that 5-hydroxycytosine is weakly mutagenic when specifically incorporated into DNA by chemical synthesis and then transferred into E. coli. (19). Thus, the results of Loeb and co-workers cannot be explained by the incorporation of the 5-hydroxycytosine modification of dCTP. It is more likely that cytosine glycol rather than 5-hydroxycytosine derivatives of dCTP were incorporated into DNA. This is compatible with the reported chemical analyses because dCTP glycols probably transformed into the 5-hydroxyl-substituted derivative before analyses (i.e. the half-life of dCyd glycols is only 50 min). Therefore, the observed mutagenesis may be attributed to the deamination of cytosine glycols to uracil glycols in DNA followed by miscoding of the latter lesions during DNA synthesis, which in turn leads to a high frequency of C to T transitions in cellular DNA (19, 20). However, the amount of cytosine glycols expected to undergo deamination in double-stranded DNA after 1 h at 37 °C may be estimated to be about 1% (the rate of deamination is 9.6 × 10\textsuperscript{−8} h\textsuperscript{−1}). In contrast, the rate of mutagenesis observed by the reverse chemical mutagenesis assay was higher (2.5%). Thus, there may have been other potentially mutagenic oxidation products of dCTP that were incorporated into DNA using this particular assay. Alternatively, it remains to be shown whether cytosine glycols are able to miscode during DNA synthesis and cause C to T transitions.

In summary, we have prepared and unambiguously identi-
H2O2-iron-induced cytosine glycols in DNA is greatly enhanced oxidation. The characterization of dCyd glycols and cytosine glycols in DNA sets down the foundation for future studies to elucidate the biochemistry and potentially mutagenic effects of cytosine oxidation.

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