Oxidative Damage to DNA Constituents by Iron-mediated Fenton Reactions

THE DEOXYCYTIDINE FAMILY*

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Damage by iron-mediated Fenton reactions under aerobic or anaerobic conditions to deoxycytidine, deoxyctydine-5′-monophosphate, d-CpC, d-CpCpC, and dCMP residues in DNA resulted in at least 26 distinguishable products. Of these, 24 were identified by high performance liquid chromatography retention times, radiolabeling, UV absorption spectra, chemical synthesis, fast atom bombardment mass spectrometry, high resolution fast atom bombardment mass spectrometry, and/or NMR. The nature of the products was qualitatively similar for each substrate except for d-CpC (and possibly d-CpCpC) under anaerobic conditions for which dissociation was uniquely present and 1-carbamoyl-1-carboxy-4-(2-deoxy-5-hydroxy-deoxycytidine was uniquely absent. Damage to dC, d-CpC, and d-CpCpC but not to dCMP or DNA was largely quenched by ethanol, indicating that iron is strongly associated only with dCMP and DNA. The presence of oxygen had little effect with dC or dCMP but had quantitative and qualitative effects with d-CpC and a significantly quantitative but not a qualitative effect with DNA. NADH could drive the Fenton reaction to cause damage to the dC family in vitro, consistent with a previous proposal that NADH was the reducing agent for the Fenton reaction in vivo (Imlay, J.A., and Linn, S. (1988) Science 240, 1302-1309). Finally, the damage spectrum of the dC family by the Fenton reaction is compared with that by ionizing radiation and chemical mechanisms leading to the formation of the 24 identified products are proposed.

Active oxygen species such as superoxide anion radical (O2−), hydrogen peroxide (H2O2), and hydroxyl radical (·OH) can damage almost all cell components, including DNA, membranes, and proteins (1). Reduction of H2O2 by reduced transition metals results in the formation of ·OH and related oxidants via the Fenton reaction (2, 3):

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{Fe}^{3+} + \text{H}_2\text{O} + \text{OH} \]  

(Dq Eq. 1)

DNA base damages induced by hypoxanthine/xanthine oxidase (4) or iron and H2O2 (5) have been identified and quantitated. Because iron chelators such as EDTA and buffers such as potassium phosphate were used in these studies, the chemistry of the Fenton reaction has probably been perturbed (6). Such chelators affect the redox potential of iron and may also scavenge oxygen radicals. Moreover, if as is generally believed, the Fenton oxidant is as reactive as is ·OH, then its diffusion distance from DNA is so short that the iron ion involved in the damage is almost certainly complexed to DNA and not to external ligands. Damage to the bases in DNA and mammalian chromatin by H2O2 and transition metals has been investigated (7–9). However, in these cases chemical hydrolysis procedures were used that might destroy, alter, or form various products (5). In order to gain further insight into the mechanisms of DNA damage by the Fenton reaction, we have therefore chosen to omit exogenous chelators and the use of chemical procedures.

Because killing of Escherichia coli by H2O2 is due to DNA damage and apparently mediated by iron with NADH as the ultimate reducing agent (10–13), we have also analyzed the degradation of the four DNA base families by iron and H2O2 under a variety of in vitro conditions. In this study of the deoxycytidine family, we have subjected 2′-deoxycytidine (dC), 2′-deoxycytidine-5′-monophosphate (dCMP), d-CpC, d-CpCpC, and DNA to iron/H2O2 under various conditions and then analyzed the resulting dC modifications.

The objectives of this and the accompanying studies were therefore extensively and systematically to study DNA damage without the use of chelators and acid hydrolysis, to identify the major stable degradation products of the dC family after Fenton reactions under a variety of conditions so as to obtain a “fingerprint” of oxidative damages that might be useful for identifying the conditions of Fenton reaction-induced DNA damage from cells subjected to oxidative stress, and to establish the chemical pathways that lead to the formation of the damaged products. Products were identified after enzymatic hydrolysis to the nucleoside level by high performance liquid chromatography (HPLC) retention times, radiolabeling, UV absorption spectra, chemical synthesis, fast atom bombardment mass spectrometry by both positive mode (FAB-MS+) and negative mode (FAB-MS−), high resolution FAB-MS, and nuclear magnetic resonance (NMR). Quantitation utilized either radiolabeling or UV absorption.

The products of ·OH attack mediated by ionizing radiation have been identified for DNA (14–18) and for cytosine (19–23). Moreover, dC damage products formed by photosensitization have been investigated by Wagner et al. (24). We therefore also are able to compare damages by these three different agents.

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The abbreviations used are: HPLC, high performance liquid chromatography; RP, reverse phase; FAB-MS, fast atom bombardment mass spectrometry; FAB-MS+, positive ion mode FAB-MS; FAB-MS−, negative ion mode FAB-MS; 5-OH-dC, 5-hydroxy-2-deoxycytidine; ddH2O, doubly distilled H2O.
Materials—dC was from Calbiochem; cytosine, dCMP, NA DH, alloxan, parabanic acid, and 2-deoxy-o-ribose were from Sigma. [1,2,5-3H]dC, [5-3H]dC, and [U-14C]dCTP were from Amersham Corp. [U-14C]dCMP was prepared from [U-14C]dCTP by snake venom phosphodiesterase digestion and purified by HPLC. H2O2 (30% solution) was from Fisher. FeCl3, FeSO4, and 1,10-phenanthroline were from Aldrich, and ethanol was from Quantum Chemical Corporation. D2O (99.9% pure) was from Cambridge isotope laboratories. Bacterial alkali phosphatase and snake venom phosphodiesterase were from Worthington; P1 nuclease was from Boehringer Mannheim; DNA polymerase I was from New England Biolabs. 5-Hydroxydeoxycytidine, cis/trans-diol-uracil, and dis-diol-uracil were gifts from Dr. Richard Wagner (Université de Sherbrooke, Sherbrooke, PQ, Canada).

Chemical Synthesis of d-CpC, d-CpCpC, and 2-Deoxy-o-ribo-β-lactone—d-CpC was synthesized by a modified triester method (25) using 5'-0-dimethoxytrityl, 3'-0-orthochlorophosphonate deoxycytidine, cymohtyresier and 3'-0-benzyloxydeoxycytidine purchased from Sigma. Radiolabeled d-CpC* (C* indicates the labeled nucleoside) was synthesized using either [5-3H]dC or [U-14C]dCTP, 4,4'-dimethoxytritylchloride, benzoyl chloride, N9-3'-dibenzoyl-deoxycytidine, and the coupling agent 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole, all of which were from Sigma. The fully protected d-CpC was deprotected by concentrated ammonium hydroxide at 50°C for 12 h followed by treatment at 80°C for 1 h in 90% acetic acid. Purification and structural confirmation were as described previously (26).

d-CpCpC was synthesized on an automated DNA synthesizer (Applied Biosystems model 380A) using the β-cyanoethyl phosphoramidite method, and then the product was purified and the structure was confirmed according to Luo et al. (26).

2-Deoxy-o-ribo-β-lactone was synthesized from 2-deoxy-o-ribose and bromine. 2-Deoxy-o-ribo-β-lactone (500 mg) was dissolved in 5 ml of doubly distilled H2O (ddH2O). 60 μl of Br2 was added dropwise, the reaction was incubated on ice for 3 h, and then unreacted Br2 was extracted with chloroform. The aqueous phase was concentrated under reduced pressure and applied to a home-packed silica HPLC column (250 × 4.6 mm), which was equilibrated with 75% ethyl acetate-16% iso-propanol-9% ddH2O. The eluate was concentrated under reduced pressure, redissolved in ddH2O, and purified on a C18 RP HPLC column for which the mobile phase was ddH2O. The identity of 2-deoxy-ribo-β-lactone was confirmed by its NMR spectrum and by FAB-MS*.

Oxygen Removal and Oxygen Concentration Measurements—The oxygen concentration in solution and the rate of O2 consumption due to the Fenton reaction were measured with an Orion Research oxygen electrode model 97-08. Air was removed from reaction solutions by sparging with N2 for 20 min prior to the addition of H2O2 and then continued sparging throughout the 30-min incubation period. Within 3 min of N2 sparging, the pO2 was below detectable levels (less than 1% of ambient).

Fenton Reaction Conditions—Substrates were exposed to Fe2+/H2O2 under aerobic conditions and to Fe3+/H2O2, Fe3+/NADH/H2O2, or Fe3+/NADH/H2O2 with or without ethanol under aerobic conditions. The damage products (except where dC was substrate) were digested to nucleosides with DNase I, snake venom phosphodiesterase, P1 nuclease, and/or bacterial alkaline phosphatase as appropriate, separated first by normal phase silica HPLC and then purified by HPLC and analyzed, all as outlined previously (26). In spite of the high resolving power of normal phase (silica) HPLC for dC degradation products, this method was not applicable for analytical studies because on-line quantitation and identification by UV absorption spectroscopy was interfered with by the presence of UV-absorbing ethyl acetate in the mobile phase. Refractometry (24) was also not amenable to quantitation because the refractive indices of standard solutions of many of the products are not known. Therefore RP HPLC was utilized for separation of the products, and UV absorption was used for on-line quantitation and preliminary identification.

FAB-MS and NMR—For positive ion mode FAB-MS, samples from dC or dCMP reactions were mixed with a glycerol matrix, which also served as an internal marker and then applied to the probe tip for bombardment by a stream of xenon gas in a Kratos Mass Spectrometer model 50. For negative ion mode FAB-MS, samples from dCMP or d-CpC reactions without enzymatic digestion were dried under reduced pressure and subjected to FAB-MS* with glycerol added as the matrix and internal marker. For NMR spectroscopy, purified samples were dried and redissolved in 5 ml of D2O, and spectra were recorded on an AM400 NMR spectrometer.

Fig. 1. Effect of pH upon level of dCMP damage. 2 mM H2O2 and 1 mM FeSO4 were added to dCMP that had been titrated with dilute NaOH to the pH values indicated. After 30 min at 25°C, the mixture was treated with bacterial alkaline phosphatase and then injected onto a C18 RP HPLC column for separation and analysis as described under “Experimental Procedures.” The amount of dCMP damaged was calculated from the disappearance of dC in the HPLC elution profile. 0.03 mM cytosine was produced at pH 4.0. Similar results were found when thin layer chromatography was used to monitor the disappearance of dC.

Other Methods—Preparation of unlabeled or βC-radiolabeled phase PM2 DNA, enzyme assays, determination of concentrations of hydrogen peroxide and iron, and HPLC were described previously (26). The concentration of PM2 DNA was estimated by UV absorption, assuming that ε0.280 = 6.5 mM−1 cm−1 dC and poly(nucleotide) concentrations were also determined by UV absorption.

RESULTS

Establishment of Reaction Conditions

2 mM H2O2 was used for Fenton reactions because this concentration gave maximal killing of E. coli (12) and should reflect both Mode I and Mode II conditions (27). Moreover, it is biologically more relevant than very high H2O2 concentrations or H2O2-generating systems, which are somewhat difficult to define, both of which are often used. Because Fe2+ was limiting (except with NADH), the concentration of iron determined the amount of damage, and, for example, the extent of damage to dCMP was proportional to ferrous concentrations up to 1 mM (data not shown). In general, the iron concentration was the minimum necessary for sufficient product characterization, but secondary damages were statistically possible under these conditions if total damage exceeded 10–20%.

To investigate the effect of pH, 1 mM dCMP was reacted with 1 mM Fe3+ and 2 mM H2O2 from pH 4 to pH 8 (Fig. 1). dCMP was chosen for this investigation because of its inherent buffering capacity. As expected for the Fenton reaction, it was more efficient at low pH, but there was a transition between pH 6 and 7. Conversely, the formation of free cytosine, one of the major dCMP damage products, increased with increasing pH (Fig. 1), perhaps indicating that high pH stabilizes an intermediate that leads to the release of cytosine. The effects of pH upon dCMP reactivity might have reflected: 1) changes of the binding of Fe3+ due to changes of the charge on dCMP (the secondary pKd of dCMP-phosphate is 6.6) (28); 2) the difference of reactivity of protonated dC and its analogs to that of unprotonated forms (the pKd of the cytosine ring is 4.3); 3) the enhanced oxidation of ferrous by oxygen above pH 6.5 (29). Parenthetically, the retention times of many dC-derived products on C18 RP HPLC columns were sensitive to pH of the elution buffers in the range 4.5–7, presumably also because they have pKd values in this range. pH 6.5 was found to give the best resolution on C18 RP HPLC columns.

Based on the above trials, 1 mM substrate was reacted with 2 mM H2O2 and 0.4–1 mM iron at pH 6.5. pH 6.5 was chosen...
because it is close to physiological pH, because ferrous is easily oxidized by oxygen at or above pH 7 (30, 31), and because the pH of 1 mM solutions of dC were 6.5-6.8 so that exogenous buffers that might alter the redox potential of iron, scavenge Fenton oxidants, and/or compete for iron binding could be avoided. FeCl₃ solutions were freshly prepared and were free of precipitate, perhaps because Fe²⁺ was still in the [Fe(H₂O)₆]³⁺ form. (The concentration of free Fe³⁺ at equilibrium with an insoluble bridged precipitate at physiological pH is estimated to be 10⁻¹⁸ M (32).) No precipitate was observed following the Fenton reactions, presumably because the ferric ions were chelated by the substrates and their damaged derivatives (33).

Characterization of the Damages
dC as Substrate—Systematic studies were carried out with either unlabeled dC (not shown), [1²',2²',5⁻³H]dC (Fig. 2A), [5⁻³H]dC (not shown), or [U⁻¹⁴C]dC (not shown), and the damaged products were traced by radiochromatograms and UV absorbance between 190 and 430 nm (26). Most of the products absorb maximally at wavelengths less than 230 nm, whereas dC has an absorption maximum at 271 nm.

The identity of the products was also determined by FAB-MS⁺, high resolution FAB-MS¹, NMR, radiolabeling, chemical synthesis, UV spectra, and HPLC retention times based on standard products that were either obtained commercially or synthesized (Table I) (26).

1-Carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was the major dC-derived product from aerobic Fenton reactions. Based on the radioactivity to absorbance ratios for the three radioactive substrates, it is concluded that this product retains all of the ¹H, ²D and C-5 tritium labels, indicating that all or part of both the sugar and base moieties are still present. The UV absorption spectrum indicates that the aromaticity is lost from this compound, so the base must be damaged, whereas the sugar is likely to be intact. Its structure was determined by its ¹H-NMR (D₂O) spectrum (Fig. 3A), FAB-MS⁺ (Fig. 4) and high resolution FAB-MS² for which the masses obtained were 278 and 278.098 by low and high resolution, respectively, (versus 278.2419 as calculated for C₉H₁₆N₃O₇). Smaller mass spectrometry peaks are consistent with fragments of this product (see legend, Fig. 4). 1-Carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide absorbs well below 205 nm with a maximum at 194 nm. Although this product is the major product from dC in the aerobic Fenton reaction, it is only a minor product in type I photosensitization of dC (24) and Fenton reactions with DNA (see below).

A prominent sugar product was 2-deoxy-o-ribofuranosyl-β-lactone. Its identity was confirmed by its UV spectrum, its retention time on RP HPLC columns, and its ¹H-NMR (D₂O) spectrum (Fig. 3B), as compared with chemically synthesized 2-deoxy-o-ribofuranosyl-β-lactone (34) and published results (35).

Slightly more damage to dC by Fe²⁺ and H₂O₂ was observed under anaerobic conditions (Table II), and the product distribution was somewhat altered (data not shown), but in general oxygen has little effect upon damage to dC (Table II).

Fig. 2. Representative HPLC chromatograms of Fenton reaction products. A, radiochromatogram of products from dC after exposure to Fe⁴⁺/H₂O₂ under aerobic conditions. 1 mM [1²',2²',5⁻³H]dC was incubated with 0.4 mM FeSO₄ and 2 mM H₂O₂ at pH 6.5 under aerobic conditions. The sample was then injected onto a C₁₈ RP HPLC column for separation and analysis. B, radiochromatogram of products from dCMP after exposure to Fe⁴⁺/H₂O₂ under aerobic conditions. 1 mM [U⁻¹⁴C]dCMP was incubated with 0.4 mM FeSO₄ and 2 mM H₂O₂ at pH 6.5 under aerobic conditions. After 30 min at 25 °C, phosphonomesters were hydrolyzed by bacterial alkaline phosphatase as described under “Experimental Procedures,” and the sample was then injected onto a C₁₈ RP HPLC column for separation and analysis. C, radiochromatogram of products from dC after exposure to Fe⁴⁺/H₂O₂ under aerobic conditions. 1 mM (nucleoside residues) [U⁻¹⁴C]dC was incubated with 0.4 mM FeSO₄ and 2 mM H₂O₂ at pH 6.5 for 30 min at 25 °C, then snake venom phosphodiesterase and bacterial alkaline phosphatase were added to convert d-CpC or damaged d-CpC to nucleosides, and then the sample was injected onto a C₁₈ RP HPLC column for separation and analysis as described under “Experimental Procedures.” D, UV chromatograph of anaerobic degradation of d-CpC. The reaction was as in C, except for purging with N₂. Inset, UV spectrum of 5-hydroxy-deoxycytidine (5-OH-dC). There are local maxima at 292, 220, and 196 nm; minima occur at 263 and 203 nm. Dotted line, A₂₃₀ nm solid line, A₂₇₈ nm. E, radiochromatogram of products from DNA after exposure to Fe⁴⁺/H₂O₂ under aerobic conditions. 1 mM PM2 DNA (nucleoside residues) labeled with [1²',2²',5⁻³H]dC was incubated with 1.0 mM FeSO₄ and 2 mM H₂O₂ at pH 6.5 aerobically. After 30 min at 25 °C, DNase I, nuclease P1, snake venom phosphodiesterase, and bacterial alkaline phosphatase were added to convert DNA or damaged DNA to nucleosides as described under “Experimental Procedures,” and the sample was then injected onto a C₁₈ RP HPLC column for separation and analysis. All radioactivity profiles were obtained by continuously collecting small samples and determining their radioactivity. Numbers correspond to the products listed in Table I.
DNA Damage by the Fenton Reaction, the Deoxycytidine Family

Table I

Identification of the products from aerobic dC-Fenton reaction

Products were obtained and characterized as described in the text. Product numbers correspond to those in Figs. 2 and 6. Whereas exact elution times vary with different HPLC columns, the order of elution and resolution was very reproducible.

| Product                                                                 | Molecular weight | Retention time | Characterized by                                                                 |
|------------------------------------------------------------------------|------------------|----------------|----------------------------------------------------------------------------------|
| 1. dR-N1-formyl-N2-glyoxylurea (or dR-isodialuric acid)                | 261              | 2.5            | FAB-MS⁺: 261 (fragments: 117 (sugar) and 145 (base))                            |
| 2. 4-Amino-1 formyl-5 hydroxy-2 oxo-3-imidazolone                       | 143              | 2.7            | FAB-MS⁺: 144                                                                     |
| 3. Parabanic acid                                                       | 114              | 2.9            | FAB-MS⁺: 115; UV spectrum and retention time were the same as standard           |
| 4. Alloxan                                                              | 142              | 3.3            | FAB-MS⁺: 143; UV spectrum and retention time were the same as standard           |
| 5. dR-urea                                                             | 176              | 3.5            | FAB-MS⁺: 177                                                                     |
| 6. 5,6-Dihydro-5,6-dihydroxy deoxycytidine                             | 261              | 3.5            | FAB-MS⁺: 262 (fragments: 117 (sugar) and 146 (base); UV spectrum                |
| 7. Unidentified                                                        | 215              | 3.6            | FAB-MS⁺: 215; has aromatic properties                                            |
| 8. Unidentified                                                        | 182              | 3.7            | FAB-MS⁺: 182; λmax = 300 nm; chemical synthesis; UV and NMR spectra; retention time was the same as reported (35) |
| 9. dR-formamide                                                        | 161              | 3.8            | FAB-MS⁺: 162 (fragment 117 (sugar)); high resolution MS⁺                           |
| 10. trans-1-carbamoyl-imidazolidone-4,5 diol                           | 161              | 4.0            | FAB-MS⁺: 162; no 117 peak; high resolution MS⁺                                    |
| 11. 2-deoxy-o-ribofuranosyl-6-lactone                                   | 132              | 4.2            | FAB-MS⁺: 133 (fragment 117 (sugar)); chemical synthesis; UV and NMR spectra; retention time were the same as reported (35) |
| 12. cis- or trans-diol-uracil (one isomer)                             | 146              | 4.5            | FAB-MS⁺: 147; UV spectrum was the same as standard                               |
| 13. cis-diol-uracil (one isomer)                                       | 146              | 4.6            | FAB-MS⁺: 147; UV spectrum and retention time were the same as standard           |
| 14. cis- or trans-diol-deoxyuridine (one isomer)                       | 262              | 4.7            | FAB-MS⁺: 263 (fragments: 117 (sugar) and 147 (base); UV spectrum was similar to that of free base |
| 15. cis- or trans-diol-uracil (one isomer)                             | 146              | 4.7            | FAB-MS⁺: 147; spectrum was the same as standard                                  |
| 16. 2,4,5-Trihydroxydeoxypyrimidine                                    | 244              | 4.8            | FAB-MS⁺: 245, 267 = 244 + Na⁺, 283 = 244 + K⁺; UV spectrum was similar to that of free base |
| 17. dR-4-amino-1 formyl-5 hydroxy-2 oxo-3-imidazolone                   | 259              | 5.0            | FAB-MS⁺: 260 (fragments: 117 (sugar) and 144 (base))                            |
| 18. Cytosine                                                           | 111              | 5.1            | FAB-MS⁺: 112; retention time and UV spectrum were the same as standard          |
| 19. 5-Hydroxyhydantion                                                  | 116              | 5.1            | FAB-MS⁺: 117; unique peak (except matrix peak) on FAB-MS⁺                         |
| 20. cis- or trans-diol-uracil (one isomer)                             | 146              | 5.2            | FAB-MS⁺: 147, 169 = 146 + Na⁺; UV spectrum was the same as standard             |
| 21. dR-trans-1-carbamoyl-imidazolidone-4,5 diol                         | 277              | 5.4            | FAB-MS⁺: 278 (fragments: 117 (sugar) and 162 (base), no peak 176, which would be a characteristic fragment of 1-carbamoyl-1-carboxy-4-(2-deoxy-o-p-erythropentofuranosyl)-glycinamide; high resolution MS⁺ |
| 22. Oxaluric acid                                                      | 132              | 6.0            | FAB-MS⁺: 133; no adduct peak; UV spectrum was different from that of 2-deoxy-o-ribofuranosyl-6-lactone |
| 23. dR-5-hydroxyhydantion                                               | 232              | 6.3            | FAB-MS⁺: 233                                                                     |
| 24. dR-biuret                                                          | 219              | 6.6            | FAB-MS⁺: 220 (fragments: 117 (sugar) and 104 (base), 242 = 219 + Na⁺, 258 = 219 + K⁺; UV spectrum was similar to that of biuret |
| 25. 1-Carbamoyl-1-carboxy-4-(2-deoxy-o-erythropentofuranosyl)-glycinamide | 277              | 7.6            | FAB-MS⁺: 278 (fragments: 117 (sugar), 162 (base only), and 176 (sugar attached to base)); high resolution MS⁺; NMR spectrum |
| 26. 5-Hydroxy-deoxycytidine*$                                            | 9.5              | Retention time and UV spectrum were the same as standard                        |
| Deoxycytidine                                                         | 228              | 10.0           | Substrate                                                                        |

$5$-Hydroxy-deoxycytidine was observed among the products of aerobic reactions of dC only after 2 weeks storage at $-20^\circ$C following the Fenton reaction.

Ethanol almost completely quenched damage to dC (Table II), indicating that ferrous ion was probably not intimately associated with dC, thus allowing the Fenton oxidant to be scavenged by the ethanol. Damage to dC by Fe$^{2+}$/NADH/H$_2$O$_2$ was also sensitive to ethanol (Table II), a surprising observation, because damage to NADH in the same reaction was resistant to ethanol. Evidently some fraction of Fe$^{2+}$ is intimately associated with NADH (but not with dC) so that ethanol-resistant radicals that damage NADH but not dC can form. Similar ethanold inhibition with Fe$^{2+}$/NADH/H$_2$O$_2$ was observed (Table II).

In all, at least 26 products were produced from Fenton reactions with dC under aerobic conditions, the majority of which were derived from modification of the base (Table I). The possibility that some of these "cytosine-derived" products might have been artifacts obtained from FAB-MS was excluded because parabanic acid, for example, was detected even before FAB-MS. The nature of the dC degradation products was generally similar under the various conditions of exposure to H$_2$O$_2$ and iron, though the yields and the product distributions varied (data not shown). All of the dC-derived products except 1-carbamoyl-1-carboxy-4-(2-deoxy-o-erythropentofuranosyl)-glycinamide have also been reported after ionizing radiation of cytosine (where appropriate) and dC (20, 36–38), and some
and the mixture was injected onto a C18 RPHPLC column were dephosphorylated with bacterial alkaline phosphatase. After the Fenton reaction, the products (and residual dCMP) phosphomonoester alter the productspectrum from that of dC. The extent of radical formation or their juxtaposition to substrate even with Fe2+/NADH/H2O2 caused less damage to dCMP than to dC. The nature of the products from this dCMP reaction was essentially the same as that from dC based on FAB-MS and the similarity of their elution profiles on RP HPLC. In contrast to the dC Fenton reactions, however, 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was one of the major products from d-CpC. There were, however, two major differences between the reactions of d-CpC and of dC with Fe2+/H2O2 under aerobic versus anaerobic conditions: 1) about twice the amount of d-CpC was damaged under anaerobic conditions compared with aerobic conditions (Table II), whereas there was no such difference with dC; 2) under anaerobic conditions, 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was not produced, whereas 5-hydroxy-deoxycytidine (5-OH-dC) was produced immediately by the Fenton reaction, unlike the case of dC, for which 5-OH-dC was observed only after 2 weeks of storage at −20 °C.

Like the case of dC but unlike that of dCMP, damage to d-CpC was severely quenched by ethanol (Table II). This observation suggests that the phosphodiester in d-CpC behaves very differently from the phosphomonoester of dCMP, probably due to both charge differences and steric hindrance. Fe3+ ion may be weakly associated with the phosphodiester group in d-CpC molecule, or alternatively, it might associate with d-CpC at the phosphodiester group and with one of the two N-3 atoms (or just the two N-3 atoms).

Like the case with dC, d-CpC damage caused by Fe2+/NADH/H2O2 and Fe3+/NADH/H2O2 was also severely quenched by the presence of ethanol (Table II). Interestingly, the formation of 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was preferred with Fe3+/NADH, as was the case for dC. The chemical basis of this phenomenon is unknown.

2 After photosensitization with 2-methyl-1,4-naphthoquinone, products 9–11, 14, 15, 18–21, and 23–26 were observed (24, 35).
In general, the nature of the d-CpC degradation products was the same as with dC under different reaction conditions with the exception of anaerobic reactions, where a novel product, 5-OH-dC, was produced, whereas 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was not formed. Damage by Fenton reactions to the 3'- and 5'-dT of d-TpT was found to be indistinguishable, so it might be expected that the same would hold for the two nucleosides of d-CpC. However, such an investigation with the 5'-nucleoside of CpC specifically labeled was not done.

DNA Damage by the Fenton Reaction, the Deoxycytidine Family

![FAB-MS of 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide](image)

**FIG. 4.** FAB-MS* of 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide. A peak at 176 (fragmented base attached to deoxyribose) has a formula of C₇H₁₄NO₄ (calculated, 176.1925; measured by high resolution, 176.0923); peak 162 (fragmented base only) has a formula of C₄H₈N₃O₄ (calculated, 162.1252; measured, 162.0518); peak 117 is presumably decomposed deoxyribose ion, which has a formula of C₅H₉O₃; peak 105 is presumably damaged base, which has a formula of C₂H₄O₃N₂. Peak 197 is presumably an adduct of 105 with glycerol. The above four peaks presumably are derived from the molecular ion of 278. Peaks 131, 153, 185, and 223 are matrix (glycerol) or its adducts with Na⁺ or K⁺ ions. The spectrum was recorded on a Kratos Mass Spectrometer model 50.

**TABLE II**

Summary of the damage to dC family by the Fenton reaction under different reaction conditions

| Substrate | Percentage of substrate dC damaged |
|-----------|-----------------------------------|
|           | Fe²⁺/aerobic | Fe²⁺/anaerobic | Fe²⁺/EtOH | Fe²⁺/NADH | Fe²⁺/NADH/EtOH | Fe³⁺/NADH | Fe³⁺/NADH/EtOH |
| dC        | 13.6 ± 0.8   | 15.9 ± 1.1   | 1.3       | 8.9 ± 0.6 | 0.7           | 10.7 ± 1.4 | 1.3           |
| dCMP      | 13.0 ± 0.6   | 15.7 ± 0.3   | 8.2       | 10.8 ± 0.6 | 10.2         | 5.2 ± 0.6  | 2.4           |
| d-CpC     | 27.3 ± 2.0   | 56.4 ± 6.2   | 1.9       | 4.7       | 1.4           | 20.4      | 3.3           |
| d-CpCpC   | (20.2)       | (22.3)       | (6.6)     | (8.7)     | ND            | (8.6)     | ND            |
| PM2 DNA   | 8.6 ± 0.8    | 14.6 ± 0.4   | 5.9       | 22.6      | 19.4          | 13.9      | 10.0          |

In general, the nature of the d-CpC degradation products was the same as with dC under different reaction conditions with the exception of anaerobic reactions, where a novel product, 5-OH-dC, was produced, whereas 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was not formed. Damage by Fenton reactions to the 3'- and 5'-dT of d-TpT was found to be indistinguishable, so it might be expected that the same would hold for the two nucleosides of d-CpC. However, such an investigation with the 5'-nucleoside of CpC specifically labeled was not done.

d-CpCpC as Substrate—d-CpCpC was initially thought to be a better model for DNA damage than dCMP or d-CpC. Surprisingly, however, the damage of d-CpCpC was quite different from that of DNA (see below) based on HPLC elution profiles and UV spectra analyses (data not shown). With Fe²⁺/H₂O₂ under aerobic conditions, the only product definitely identified from UV absorption chromatograms was unaltered cytosine base. Peaks at positions corresponding to dR-formamide, trans-1-carbamoyl-imidazolidone-4,5-diol 2-deoxy-β-D-ribonolactone, and possibly 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide were the remaining major products of d-CpCpC under aerobic conditions.

Unlike the case for DNA (see below), oxygen did not play a major role in the damage of d-CpCpC, and a significant amount of d-CpCpC damage was quenched by ethanol (Table II). Fe²⁺/NADH/H₂O₂ and Fe³⁺/NADH/H₂O₂ induced the same amount of damage (Table II), in contrast to DNA, for which Fe²⁺/NADH/H₂O₂ induced much more damage than Fe³⁺/NADH/H₂O₂. All of these differences probably stem from the difference

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3 R. Chattopadhyaya, R. Jin, E. Henle, Y. Luo, and S. Linn, unpublished observations.
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3 ml of 140 μM calf thymus DNA (nucleotide residues), 70 μM FeSO₄, and 140 μM NADH where indicated, were mixed in a dialysis bag and then dialyzed under N₂ against 500 ml of 0.8% NaCl for 24 h with the dialysis solution changed every 6 h. The final concentrations of Fe²⁺ inside the dialysis bag were measured with 1,10-phenanthroline as described under “Experimental Procedures.”

| Components present | Fe²⁺ inside the dialysis bag |
|--------------------|-----------------------------|
| DNA/FeSO₄          | 4.2                         |
| DNA/FeSO₄/NADH     | 13.4                        |

**Table III**

| Substrate                  | Percentage of dC damage with Fe²⁺/H₂O₂/air | Percentage of dC damage with Fe³⁺/NADH/H₂O₂/air |
|----------------------------|-------------------------------------------|-------------------------------------------------|
|                            | N₂ sparging Ethanol  NADH     NADH/ethanol | NADH/ethanol                                    |
| dC                         | 117                        9.6        65         7.9       |
| dCMP                       | 121                        63         83         94        |
| d-CpC                      | 207                        7.0        17         30        |
| d-CpCpC                    | 110                        33         43         86        |
| DNA                        | 170                        68         263        86        |

**Table IV**

by which iron binds to d-CpCpC and to DNA. From simple model building it is easily seen that a d-CpCpC molecule can bend so as to utilize two phosphates to bind one iron ion. However, duplex DNA cannot adopt this conformation. Therefore, it was concluded that d-CpCpC was not a good model for duplex DNA damage studies, except that the last column (NADH/ethanol) is relative to that with NADH without ethanol present.

dC in Duplex DNA as Substrate—Systematic studies of the degradation of the dC moiety in duplex DNA were carried out with DNA radiolabeled with [1',2',5'-H]dCTP by nick translation (40). With this substrate, about 9 and 14% of the dC moieties were damaged under aerobic and under anaerobic conditions with Fe²⁺/H₂O₂, respectively (Fig. 2E, Table II), a level similar to that with d-CpC. However, 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was not a major product from DNA, unlike the case of d-CpC, where it was. The majority of damage to the dC moiety of duplex DNA was not ethanol-quenchable, as is the case for DNA nicking (27).

In the presence of NADH, much more damage to the dC moiety in DNA occurred than to dC, dCMP, d-CpC, or d-CpCpC. There was a more than two-fold increase with Fe²⁺/NADH/H₂O₂ than with Fe²⁺/H₂O₂, respectively (Table II), a level similar to that from dC, dCMP, and d-CpC. However, 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was not a major product from DNA, unlike the case of d-CpC, where it was. The major product of damage to the dC moiety of duplex DNA was not ethanol-quenchable, as is the case for DNA nicking.

Comparison of Damages among the Various Substrates—The nature of the dC degradation products from DNA were basically the same as that from dC, dCMP, and d-CpC based on the analysis of their elution profiles and UV spectra, although the yields and the product distributions differed somewhat. However, dC, dCMP, d-CpC, nor d-CpCpC was a perfect model for DNA because the damage enhancement in the presence of NADH is unique to duplex DNA. Various comparisons of the degree of damage to the members of the dC family after Fenton reactions with d-CpC, in contrast to the analogous aerobic Fenton reaction where the opposite result was observed.

**Fig. 5. Proposed effect of oxygen on the d-CpC Fenton reactions.** 5-OH-dC but not 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was detected in anaerobic Fenton reactions with d-CpC, in contrast to the analogous aerobic Fenton reaction where the opposite result was observed.
is possible either that the oxygen produced from the decomposition of H$_2$O$_2$ was not completely removed by N$_2$ flushing or that H$_2$O$_2$ can substitute for oxygen in reacting with carbon-centered radicals. Alternatively, oxygen may not directly take part in the damage of these substrates during Fenton reactions.

In order to distinguish among these hypotheses, the oxygen concentration was measured during the Fenton reaction of Fe$^{2+}$ with H$_2$O$_2$ under aerobic conditions. In the absence of substrate, the Fenton reaction produced oxygen as expected (Table V); however, the oxygen concentration decreased when substrates were present, and a more rapid decrease in the oxygen concentration occurred with dC or d-CpC than with dCMP (Table V), although dC and dCMP were damaged to the same extent (Table II). This result may indicate that the dCMP radicals formed are not accessible to oxygen so that less oxygen
is consumed. The observation that more oxygen is consumed by dC or d-CpC is consistent with an oxygen requirement for the formation of 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide, because it is the major product from both dC and d-CpC but only a minor one from dCMP aerobic Fenton reactions (see “Discussion”).

**DISCUSSION**

dC is the most sensitive of the four deoxynucleosides to aerobic Fenton reactions. 70% of the dC is damaged with 1 mM Fe²⁺/2 mM H₂O₂ present, whereas 55% of dT, 31% of dA, and 28% of dG are damaged (39). Likewise, dCMP is the most sensitive of the four 5-mononucleosides; about 96% of dCMP is damaged with 1 mM Fe²⁺/2 mM H₂O₂ at pH 4.0 under aerobic conditions, whereas 55% of dTMP, 17% of dAMP, and 22% of dGMP are damaged (39). Similarly, d-CpC is the most sensitive of the four d-Nps; about 26% of d-CpC is damaged with 1 mM Fe²⁺ and 2 mM H₂O₂ under aerobic conditions, whereas 18% of d-TpT, 16% of d-GpG, and 12% of d-ApA are damaged. On the other hand, about 9% of the dC in DNA was damaged under aerobic Fenton reactions (Fig. 2E), compared with 12% of the dT, 6% of the dA, and 8% of the dG under the same conditions (39). The greater sensitivity of dC and d-CpC to iron/H₂O₂ compared with the other three base families is not reflected in the dC moiety in DNA.

It is clear from the product profiles (Table I) and previous studies (17, 24) that the main attack site of oxidative radicals on dC is the C-5-C-6 bond. Based on quantum chemical considerations, Pullman and Pullman (42) predicted that the C-5-C-6 bond has the highest electron-donating capacity within cytosine or cytosine nucleosides, leading Van de Vorst and Westhof (43) also to argue that the C-5-C-6 bond is the most likely target of oxidative radicals such as the OH radical. From the present study it also appears that the C-5-C-6 bond is the major site of attack for radicals generated by the Fenton reaction.

It appears that 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide is produced mainly by freely diffusible ‘OH or a similar radical because 1) it was the major dC-derived product (Fig. 2A) and such a diffusible radical is presumably the damaging radical in these reactions; 2) it was a minor product with dCMP (Fig. 2B), for which freely diffusible ‘OH is apparently not the major damaging species. Moreover, formation of 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was not observed under anaerobic reactions with d-CpC, suggesting that oxygen is required for the formation of this product.

The damage to d-CpC and DNA was enhanced by at least 50% under anaerobic versus aerobic conditions, and in addition, 5-OH-dC was produced only with anaerobic d-CpC Fenton reactions. Conversely, 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide was not detected under these conditions. Oxygen may participate in a way to enhance formation of some degradation products (e.g. 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide) or to inhibit formation of others (e.g. 5-OH-dC formation from d-CpC). One plausible mechanism for the oxygen inhibition is that under anaerobic conditions, a carbon-centered radical on DNA or d-CpC is formed due to the Fenton reaction and the nascent Fe³⁺ or another Fe³⁺ ion reacts with this carbon-centered radical to regenerate Fe²⁺ ion, which then undergoes a second cycle of Fenton reaction to cause further damage to DNA or d-CpC. However, if O₂ were present, it could react with the carbon-centered radical and a peroxy radical R-OO⁻ would form, which would then lead to the formation of different products, such as 1-carbamoyl-1-carboxy-4-(2-deoxy-β-D-erythropentofuranosyl)-glycinamide. The peroxy radical might also oxidize Fe²⁺, thus leading to less overall damage. A scheme that summarizes these suggestions is shown in Fig. 5. Why the formation of 5-OH-dC would be substrate-dependent is unclear, but it might reflect a unique type of complex with iron ions.

Equilibrium dialysis indicated that a ternary complex is formed among DNA, Fe³⁺+, and NADH. Such a complex may also exist among DNA, Fe³⁺+, and NADH, although it is almost impossible to directly determine the equilibrium binding constants for DNA, Fe³⁺+, and NADH because Fe³⁺+ will be reduced by NADH before an equilibrium is established. The enhanced damage (compared with Fe²⁺/H₂O₂) due to the presence of NADH occurred only with DNA (see Table II and Table V), not with dC, dCMP, d-CpC, and d-CpCpC, suggesting that the binding of NADH (and iron) to DNA allows NADH to efficiently drive the Fenton reaction to cause damage. This observation suggests that an NADH-iron complex might intercalate into DNA or bind to one of the DNA grooves through both electrostatic and van der Waals’ interactions. However, the NADH/iron complex cannot intercalate into d-CpC or d-CpCpC because these substrates cannot provide efficient base stacking and do not contain stable secondary structures.

A comprehensive scheme of alternative pathways that lead to the formation of each of the products detected with the dC family is proposed in Fig. 6. This scheme is obviously tentative and somewhat speculative, so it should be taken as a working model for future experiments. Measurement of the products found in the scheme might be used as an index of oxidative attack upon DNA in living tissues or in isolated cells (44). Moreover, comparisons of the relative amounts of some of the products might be useful to discern the in vivo reaction environments under different conditions of oxidative stress, a final goal of these studies.

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