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Endogenous oxidative damage of deoxycytidine in DNA

(5-hydroxy-2'-deoxycytidine/5-hydroxy-2'-deoxyuridine/deoxyuridine glycol/mutagenesis/carcinogenesis)

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ABSTRACT **Three major oxidation products of 2'-deoxycytidine (dC)—5-hydroxy-2'-deoxycytidine (oh^{5}dC), 5-hydroxy-2'-deoxyuridine (oh^{5}dU), and 5,6-dihydroxy-5,6-dihydro-2'-deoxyuridine (dUg)—were analyzed from enzymatically hydrolyzed DNA with reversed-phase high-performance liquid chromatography coupled to electrochemical detection. oh^{5}dC and oh^{5}dU can be detected with high sensitivity (50 fmol) and selectivity (0–0.2 V) from hydrolyzed DNA. dUg is not electrochemically active but can be measured by dehydrating it into oh^{5}dU. The quantities of oh^{5}dC, dUg, and oh^{5}dU in untreated commercial-grade calf thymus DNA are 10, 10, and 0.75 fmol/μg of DNA, respectively. These levels increased substantially when calf thymus DNA was exposed to ionizing radiation, H_2O_2 alone, H_2O_2 and combinations of Fe^{3+} or Cu^{2+} and ascorbate, near-UV light (365 nm), near-UV light in the presence of menadione, and OsO_4, indicating that oh^{5}dC, oh^{5}dU, and dUg are major oxidative DNA damage products. The steady-state levels of these products were determined from freshly extracted rat tissues and ranged from <0.5 fmol/μg of DNA for oh^{5}dU to about 10 fmol/μg of DNA for oh^{5}dC and dUg in liver and kidney and 22 fmol/μg of DNA for oh^{5}dC in brain. The levels of oxo^{8}dG were also determined and in general were somewhat lower than the levels of oh^{5}dC. These findings reinforce the link between DNA damage induced by oxidative metabolism and spontaneous mutagenesis leading to cancer and aging.**

The utilization of oxygen by aerobic organisms generates large amounts of oxidants, including O_2^-, H_2O_2, ·OH, and $^1O_2$, which in turn alter target biomolecules such as lipid, protein, and DNA (1–5). The production of potentially lethal doses of oxidants is counterbalanced by a wealth of antioxidants that are, in part, made up of small molecule scavengers such as glutathione, ascorbate, urate, bilirubin, ubiquinol, β-carotene, and tocopherol as well as specialized proteins such as superoxide dismutase and glutathione peroxidases (1, 2, 6, 7). The balance of oxidants and antioxidants favors the former since oxidative damage persists in organisms even under normal unstressed conditions (3–8). The metabolic rate, which is related to the oxidative DNA damage rate (8), may be an important factor in the average life span of different organisms (9). Aging and the variety of degenerative diseases associated with it such as cancer may be due in good part to oxidative damage (3–5, 8–10). Oxidative DNA damage mediated by phagocytic cells may explain in part the high incidence of cancer in areas of chronic inflammation (11, 12). In addition, a large body of clinical and epidemiological studies indicates that the level of antioxidants from the diet have a protective effect against many age-related disorders including cancer (13–15).

Oxidative DNA damage *in vivo* likely arises by the initial reaction of OH radicals generated by metal ions bound to DNA and cellular oxidants (i.e., O_2^- and H_2O_2), which are produced during normal metabolism or oxidative stress (16). Hydroxyl radicals are also the principal reactive species in the deleterious effects of ionizing radiation (17). Both sources of OH radicals are known to induce mutagenesis and carcinogenesis (18, 19). The molecular steps in ·OH-induced mutagenesis are not fully understood due in part to the multitude of different modifications that may be formed in DNA from OH radicals (17, 20, 21). The formation of only a few of these lesions including 5,6-hydroxy-5,6-dihydrothymidine (dTg; dT glycol) and 5-hydroxymethylthymidine from dT oxidation and 8-oxo-7,8-dihydro-2'-deoxyguanosine (oxo^{8}dG; 8-hydroxy-2'-deoxyguanosine) from dG oxidation has been observed in nuclear DNA after cells or whole rats are exposed to near or greater than lethal doses of mutagens (22–24). The steady-state level of oxo^{8}dG arising from oxidative metabolism of unstressed organisms appears to be the highest so far determined: about 1 oxo^{8}dG per 100,000 dG, or about 10^6 total oxidative lesions per genome per rat cell (4). The steady-state level of dTg, 5-hydroxymethyl-2'-deoxyuridine, and oxo^{8}dG is maintained by specific DNA repair processes, which have been identified for these lesions in bacterial and mammalian cells (25–27). The detection of this type of DNA damage in tissue culture spent medium or urine also indicates that organisms have efficient repair systems against oxidative DNA damage (3, 28–30). From the levels of dTg and oxo^{8}dG as well as the corresponding nucleotide bases in urine, total oxidative damage to DNA is estimated to be about 10^5 hits per cell per day in the rat, which suggests that oxidative damage may be the major contributor to endogenous DNA damage (3–5).

The implications of the large endogenous oxidative DNA damage for mutagenesis, carcinogenesis, and aging have prompted us to extend this work to other forms of oxidative DNA damage. The development of a sensitive assay in this work using HPLC coupled to electrochemical detection (EC) has made it possible to measure three other DNA lesions derived from dC oxidation: 5,6-dihydroxy-5,6-dihydro-2'-deoxyuridine (dUg; dU glycol), 5-hydroxy-2'-deoxycytidine(oh^{5}dC), and 5-hydroxy-2'-deoxyuridine (oh^{5}dU).

MATERIALS AND METHODS

Synthesis of Standards. The synthesis of oh^{5}dC and oh^{5}dU was carried out as reported for the riboside (31). dUg was obtained by oxidation of dU with $KMnO_4$ as reported for thymidine (32). The identity of each standard was confirmed by GC–MS analysis of the trimethylsilyl derivatives. UV spectra data were as follows: oh^{5}dU, ε(max, 278 nm) = 7335 $M^{-1}\cdot cm^{-1}$ and oh^{5}dC, ε(max, 292 nm) = 6025 $M^{-1}\cdot cm^{-1}$ at

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Abbreviations: oh^{5}dC, 5-hydroxy-2'deoxycytidine; oh^{5}dU, 5-hydroxy-2'-deoxyuridine; dUg, 5,6-dihydroxy-5,6-dihydro-2'-deoxyuridine (dU glycol); dCg, 5,6-dihydroxy-5,6-dihydro-2'-deoxycytidine (dC glycol); dTg, 5,6-hydroxy-5,6-dihydrothymidine (dT glycol); oxo^{8}dG, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-hydroxy-2'-deoxyguanosine); EC, electrochemical detection; SPE, solid-phase extraction.

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pH 7. Radiolabeled oh^{5}dC, oh^{5}dU, and dUg were synthesized as above except with [^{3}H]dC obtained by dephosphorylation of [1′,2′,5-^{3}H]dCTP (70 Ci/mmol; 1 Ci = 37 GBq; Amersham) and [^{3}H]dU obtained by deamination of [^{3}H]dC (33).

Extraction of DNA from Biological Sources. Male Fisher 344 rats aged 3–28 months were purchased from Simonson Laboratories (Gilroy, CA) and from the National Institute of Aging. Animals were anesthetized by exposure to CO_2 gas for 1–2 min followed by decapitation. Liver, kidney, and brain were homogenized with 10 volumes of buffer solution containing 0.15 M NaCl, 10 mM Tris·HCl (pH 7.4), and 1 mM EDTA. Nuclei were isolated by centrifugation at 3000 rpm (1100 × *g*) for 15 min. Human leukocytes from four volunteers aged 24–62 were obtained by a mild and rapid procedure from freshly drawn blood (34). Rat nuclei and human leukocyte DNA were extracted with an automated DNA extractor (Applied Biosystems) using Applied Biosystems reagents. Samples were dried and frozen until analysis.

Analysis of dC Oxidation Products from DNA. Analysis of oh^{5}dC, oh^{5}dU, and dUg involved digestion of DNA by enzymatic hydrolysis, separation into two polar fractions by solid-phase extraction (SPE), and quantitation by HPLC–EC; analysis of dUg involved additional steps to convert it into oh^{5}dC. Quantitation was based on the electrochemical signal and the recovery of the corresponding ^{3}H-labeled standard, which was added prior to sample workup. Samples containing 0.25–1.0 mg of DNA were denatured by heating for 3 min at 90°C in 1 ml of bis-Tris·HCl (5 mM, pH 7.0) and EDTA (0.1 mM). Radiolabeled standards added were [^{3}H]dUg (0.1 μCi), [^{3}H]oh^{5}dC (0.01 μCi), and sometimes [^{3}H]oh^{5}dU (0.01 μCi). Sodium acetate buffer (20 mM, pH 4.8) and P1 nuclease (100 μg/ml; Sigma) were then added, and the solution was incubated for 30 min at 37°C. The pH was adjusted with Tris·HCl buffer (100 mM, pH 8.0); then bacterial alkaline phosphatase (20 units/ml; Boehringer Mannheim), snake venom phosphatase (34 μg/ml; Boehringer), and $MgCl_2$ (10 mM) were added; and the mixture was incubated at 37°C for an additional 1.5 h. The samples were separated on C18/OH SPE disposable cartridges (Analytichem International, Harbor City, CA) into two fractions by stepwise elution of 2 ml of phosphate buffer (25 mM, pH 4.0), which removed the dUg fraction, followed by 4 ml of the same buffer, which removed the fraction containing both oh^{5}dC and oh^{5}dU. These fractions were taken to dryness. The first fraction containing dUg was further purified by HPLC using a Supelcosil LC-18 (10-μm particle size) column (10 mm × 25 cm; Supelco, Bellefonte, PA) at a flow rate of 2 ml/min with 2% MeOH in water. Five percent of the eluant was split into a radiometric detector, and the corresponding ^{3}H-labeled dUg peak (2.5–3.0 ml) was collected. To this volume was added 250 μl of collidine, and the mixture was heated at 80°C for 30 min, extracted twice with diethyl ether, and concentrated to dryness until analysis. Analysis of dUg (the first SPE fraction) and of oh^{5}dC together with oh^{5}dU (the second SPE fraction) was carried out by isocratic HPLC using a Supelcosil LC-18-DB (3-μm particle size) column (4.6 mm × 15 cm; Supelco) at a flow rate of 1 ml/min with acetate buffer (25 mM, pH 4.5) connected in series to electrochemical, UV, and radiometric detectors. The HPLC system consisted of a 712 WISP autoinjector (Waters), a model M510 pump (Waters) equipped with two pulse dampeners (SSI), a model 5100A Coulochem electrochemical detector (ESA) equipped with a 5010 dual flowcell, a model 770 Spectroflow multi-wavelength UV detector (Kratos Analytical Instruments), and a radiometric detector (Radiomatic Instruments and Chemicals, Tampa, FL). The data from HPLC analysis was digitized by a Nelson 760 analytical interface (Cupertino, CA) and processed by Perkin–Elmer/Nelson Analytical TURBOCHROM data acquisition software on an IBM PS/2 model 70 computer.

Treatment of DNA Samples with Oxidants. Calf thymus DNA (Sigma) was treated with Chelex resin, neutralized with HCl, precipitated by the addition of acetate (0.3 M) and ethanol (2.2 vol), and dried on a vacuum pump before use. The concentration of DNA in aqueous solution at pH 7 was determined by UV absorption at 260 nm (OD = 1.0 for 50 μg of DNA). Solutions of DNA (0.5 mg/ml) in doubly distilled phosphate-buffered water (25 mM, pH 7) were subjected to the following conditions: (*i*) 0–125 grays of γ-radiation, dose rate = 10 grays/min (samples were bubbled with air before and during irradiation); (*ii*) combinations of H_2O_2 (2.8 mM), $FeCl_3$ (50 μM), or $CuSO_4$ (50 μM) and ascorbate (200 μM) incubated at 37°C for 1 h; (*iii*) near-UV irradiation for 20 min at 5 cm with a 200-W Blak-Ray lamp (UV Photoproducts, San Gabriel, CA); (*iv*) condition *iii* plus 0.4 mM menadione (Aldrich); and (*v*) 0.2% OsO_4 (Aldrich) at 37°C for 1 h. After each of these reactions, DNA was purified twice by ethanol precipitation before analysis.

RESULTS

Analysis of oh^{5}dC and oh^{5}dU in DNA. The measurement of trace levels of oh^{5}dC and oh^{5}dU from hydrolyzed DNA is possible because of their ideal electrochemical properties. The oxidation of these compounds takes place within a narrow range of voltages (0.1 V) and at low oxidation potentials (0.07 for oh^{5}dC and 0.15 for oh^{5}dU). The sensitivity of their detection is about 50 fmol for a vertical signal-to-noise ratio of 2–3. The detection of oh^{5}dC and oh^{5}dU from 410 μg of hydrolyzed calf thymus DNA was well above the detection limit (Fig. 1*a*). The amount of oh^{5}dC was easily determined from freshly extracted DNA; however, the amount of oh^{5}dU was 20- or more fold lower, and its analysis was not feasible from these samples. The identification of both peaks was based on cochromatography with authentic oh^{5}dC and oh^{5}dU and comparison of the electrochemical

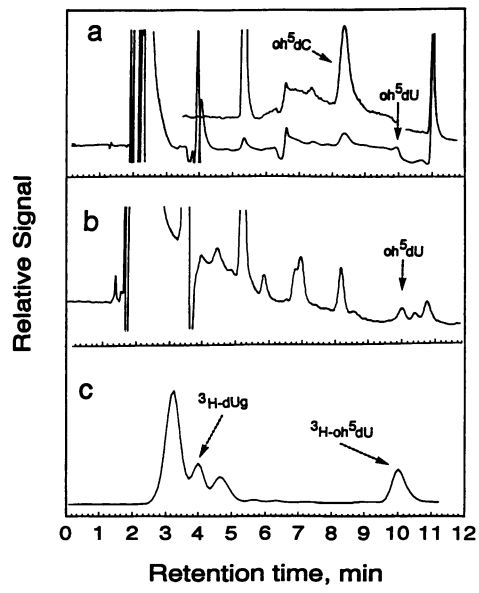

FIG. 1. Analysis of dUg, oh^{5}dC, and oh^{5}dU by HPLC–EC from untreated calf thymus DNA. (*a*) The traces show the HPLC–EC analysis of oh^{5}dC and oh^{5}dU (from 410 μg of DNA). The detection of oh^{5}dC was achieved on the first EC cell set at 0.0–0.10 V (upper chromatogram) and that of oh^{5}dU was achieved on the second cell with a window of 0.10–0.20 V (lower chromatogram). (*b* and *c*) Analysis of dUg. (*b*) HPLC–EC of oh^{5}dU (410 μg of DNA). (*c*) Simultaneous trace of the radioactivity of the radiolabeled standard.

response as a function of voltage, a characteristic property of each compound.

Analysis of dUg in DNA. The analysis of dUg, which is not EC active, was accomplished by dehydrating it to oh^{5}dU. Fig. 1 *b* and *c* depicts the final analysis of oh^{5}dU derived from dUg in hydrolyzed calf thymus DNA. It shows the detection of ^{3}H-labeled and EC-active peaks from the same run. The EC-active peak at 10 min was identified as oh^{5}dU because it has the same electrochemical profile as authentic oh^{5}dU, it coelutes with oh^{5}dU, and it arises uniquely from the purified dUg fraction. The additional peaks in the EC chromatogram that accompany oh^{5}dU were consistently observed in every analysis, including those without DNA, and thus appear to arise from the reaction mixture. The overall efficiency of dehydration was 5–15% when HPLC-purified dUg was heated at 80°C for 30 min in the presence of collidine. Attempts to improve the conversion of dUg into oh^{5}dU by changing the reaction conditions by adding different buffers (Tris·HCl, phosphate, acetate, and borate), strong acid or base (pH 2–10), or catalytic amounts of toluenesulfonic acid, imidazole, or pyridine resulted in the disappearance of dUg in most cases, but the yield of oh^{5}dU was greatly reduced (<1%). The analysis of dUg from DNA probably includes all four possible diastereoisomers. Although *trans*-(−)- and -(+)-dUg are excluded during purification of *cis*-(−)- and -(+)-dUg in the assay, the trans isomers are probably included in our measurement of total dUg because they transform into cis isomers, similar to dTg (35), after initial DNA hydrolysis.

Formation of dC Oxidation Products from Oxidized DNA. The levels of dUg, oh^{5}dC, and oh^{5}dU in DNA increased substantially above steady-state levels when calf thymus DNA was exposed to a number of conditions that are known to induce the oxidation of DNA components (Table 1). Ionizing radiation induced the formation of dUg (or dCg), oh^{5}dC, and oh^{5}dU linearly with dose in the range of 0–125 grays (data not shown). The *G* values (molecules of product per 100 eV absorbed) calculated from the dose–response on the spontaneous release of nucleotide bases ($G = 0.3$) (36) were 0.021, 0.025, and 0.036 for dUg, oh^{5}dC, and oh^{5}dU, respectively. The formation of these three products involves deamination and/or dehydration from dCg, which is likely the initial modification of dC induced by OH radicals. We expect that oh^{5}dC and oh^{5}dU are mainly produced by these processes in DNA rather than in solution once DNA is hydrolyzed. The extent of deamination and dehydration of dCg in aqueous solution can be estimated from the radiolysis of monomeric dC, which gives dUg and oh^{5}dC in a ratio of 2.5:1, respectively. Therefore, since the ratio of dUg to oh^{5}dC from 5,6-dihydroxy-5,6-dihydro-2′-deoxycytidine (dCg; dC-glycol) in DNA (1:1) is 2.5-fold higher than that from dCg in solution (2.5:1), then 70% [(2.5/3.5) × 100] of the total oh^{5}dC in DNA analysis must come from dehydration of dCg within DNA. In contrast, oh^{5}dU is not formed by radiolysis of monomeric dC, and neither dUg nor oh^{5}dC transforms into oh^{5}dU under neutral aqueous conditions; thus, all of oh^{5}dU in DNA analysis must arise from sequential deamination and dehydration of dCg in DNA. Like ionizing radiation, Fenton reactions with 2.8 mM H_2O_2 alone or combined with 50 μM Fe^{3+} or 50 μM Cu^{2+} and/or 200 μM ascorbate led to the formation of dUg, oh^{5}dC, and oh^{5}dU in DNA (Table 1). The greater reactivity of Cu^{2+} toward dC oxidation may in part be due to the ability of Cu^{2+} to chelate with dC residues in DNA (37). Irradiation with near-UV light alone led to modest increases of only oh^{5}dU and oh^{5}dC, whereas in the presence of menadione, all three products were found to increase substantially (Table 1). The reaction of OsO_4 with DNA predominantly resulted in the formation of oh^{5}dU.

Table 1. *In vitro* oxidation of DNA oxidative products

Oxidant added	Yield, fmol/μg of DNA		
	oh^{5}dC	dUg	oh^{5}dU
None	10	10	0.75
γ-Radiation (10 gray)	120	100	150
H_2O_2	27	47	15
H_2O_2/Fe^{3+}	58	64	50
H_2O_2/Fe^{3+}/ascorbate	520	350	620
H_2O_2/Cu^{2+}	1500	1100	470
H_2O_2/Cu^{2+}/ascorbate	4300	2510	870
UV (365 nm)	32	10	6
UV + menadione	240	210	780
OsO_4	10	57	360

Calf thymus DNA was exposed to different oxidants according to the conditions stated in *Materials and Methods*. The solutions were then analyzed for dUg, oh^{5}dC, and oh^{5}dU by HPLC–EC. Calf thymus DNA was commercial, and it is not known whether some of the oxidized bases were formed during its isolation.

Steady-State Levels of dC Oxidation Products in Freshly Extracted DNA. The steady-state levels of dC oxidation products in freshly extracted DNA isolated from rat liver, kidney, and brain were examined (Table 2). Both oh^{5}dC and dUg were found to be present in these tissues; however, the levels of oh^{5}dU in most samples were undetectable (<0.5 fmol/μg of DNA). The amount of oh^{5}dC in rat brain (22 fmol/μg of DNA) was twice as high as that in liver and kidney (10 fmol/μg of DNA). The levels of dUg were in the range 8.5–14.6 fmol/μg, with brain > kidney > liver. There was no consistent increase in the levels of dUg or oh^{5}dC in rat liver or kidney as a function of age. For oh^{5}dC in rat brain, we did observe some increase in the level of oh^{5}dC between 5-month-old (14.7 ± 1.3; $n = 3$) and 28-month-old (17.8 ± 0.4; $n = 3$) rats.

It is unlikely that dUg and oh^{5}dC are produced during tissue homogenization or extraction of DNA since oh^{5}dU is not detected in most samples, whereas this product is equally important when DNA is exposed to oxidizing conditions. The presence of oh^{5}dU in commercial-grade calf thymus and in human leukocytes indicates that DNA does undergo oxidation to some extent in these samples. However, the amount of artifactual formation in rat DNA from all sources should not exceed, and in fact should be lower than, that in human leukocyte DNA (Table 2). Self-radiolysis, which may arise from the radiolabeled standards, does not induce the formation of oh^{5}dC or oh^{5}dU. The addition of radioactivity to solutions of dC under conditions identical to those used for DNA analysis did not induce the formation of oh^{5}dC or oh^{5}dU, and direct analysis of DNA hydrolysates without adding radiolabeled standards resulted in similar yields of dC oxidation products.

DISCUSSION

This work describes the analysis of oxidative dC lesions in DNA by enzymatic hydrolysis and HPLC–EC. This method is well suited for the analysis of DNA lesions that have low oxidation potentials. The four major unmodified deoxynucleosides are unresponsive at voltages below about 0.5 V. Furthermore, most of the known oxidation products of deoxynucleosides are not sensitive to EC, mainly because oxidation results in saturation of purine and pyrimidine ring systems. Exceptions to this in the order of their electrochemical oxidation potentials at half maxima are 8-oxo-7,8-dihydro-2′-deoxyadenosine (0.55 V), oxo^{8}dG (0.19 V), oh^{5}dU (0.15 V), and oh^{5}dC (0.07 V). Therefore, all of these products may be detected from enzymatic digests of DNA with 50-fmol sensitivity and good selectivity by HPLC–EC. The detection of oxo^{8}dG from DNA has become a standard analysis of endogenous DNA damage (24). The measurement of endog-

Table 2. Yields of oxidative DNA products from biological samples

Sample	Yield, fmol/μg of DNA			
	oh^{5}dC (*n*)	dUg (*n*)	oh^{5}dU (*n*)	oxo^{8}dG (*n*)
Calf thymus	10 ± 2.5 (3)	10 ± 4.0 (3)	0.75 ± 0.25 (3)	48 ± 5.0 (3)
Rat liver	10 ± 3.5 (9)	8.5 ± 3.5 (6)	<0.5	8.6 ± 0.6 (6)
Rat kidney	9.9 ± 4.0 (9)	10.3 ± 4.0 (7)	<0.5	8.2 ± 1.5 (6)
Rat brain	22.6 ± 3.4 (16)	14.6 ± 4.5 (6)	<0.5	5.0 ± 1.2 (6)
Human leukocytes	3.1 ± 1.6 (4)	6.2 ± 4.6 (4)	2.1 ± 1.8 (4)	3.5 ± 2.1 (4)

DNA at 0.5–1.0 mg/ml was analyzed by HPLC–EC. The average levels of dUg and oh^{5}dC are reported with standard deviations, and the number of animals is given in parentheses.

enous dC oxidation products in DNA requires larger amounts of DNA and longer sample workup than oxo^{8}dG. It is necessary to fractionate the very polar dC oxidation products from the other nucleosides in DNA hydrosylates in order to simplify HPLC–EC analysis using isocratic conditions. Large amounts of DNA (≈500 μg) are needed for the analysis of oh^{5}dU because of its low steady-state levels in DNA and for the analysis of dUg because of its poor conversion into oh^{5}dU.

An alternative approach for the analysis of oxidative lesions in DNA has been GC–MS (38–40). The protocol involves hydrolyzing DNA to nucleotide bases by strong acid (150°C for 90 min) followed by trimethylsilylation and GC–MS analysis (38–40). There is an apparent discrepancy on the quantitative aspects of HPLC–EC compared to the GC–MS analysis of dC oxidation products. The levels of 5-hydroxycytosine and 5-hydroxyuracil reported by GC–MS analysis were generally much higher than the levels of oh^{5}dC, oh^{5}dU, and dUg obtained by HPLC–EC analysis (acid hydrolysis transforms all three nucleosides into the above base derivatives). The steady-state levels in commercial-grade calf thymus DNA by GC–MS (total = 140 fmol/μg of DNA) (40) are 8 times higher than those by HPLC–EC (total = 25 fmol/μg of DNA). (Some of this, however, could be due to varying degrees of preexisting DNA oxidation.) The *G* values by GC–MS (total = 0.24) (39) are a factor of 3 higher than we find by HPLC–EC (total = 0.082). Fenton reactions using identical oxidation procedures (Table 1) (40) also show a marked excess from the GC–MS method. The reason for the above discrepancy could in part be due to artifactual oxidation of DNA during the 150°C hydrolysis or the derivatization steps in the preparation of samples for GC–MS. On the other hand, with the enzymatic hydrolysis we optimized the release of dUg, oh^{5}dU, and oh^{5}dC by using an excess of enzyme. For comparison, oh^{5}U and oh^{5}C are completely digested from polyribosides (31), and dTg is nearly completely digested (100% or 80%, depending on the isomer) from modified dTpA (41) by using a digestion procedure similar to the one in this work.

Hydroxy radicals react with dC mainly by addition to the pyrimidine 5,6 double bond (17). The resulting OH adduct radicals appear to account for the formation of a number of stable dC oxidation products via intermediate hydroperoxy radicals and hydroperoxides (17). The products are similar to those of dT (i.e., glycol, hydantoin, and formamide derivatives), with the exception of unique products for dC, which arise from rearrangement of the pyrimidine ring (21). Unlike dTg and dUg, however, dCg is not stable in aqueous solution, and it rapidly transforms into dUg by deamination and partially into oh^{5}dC by dehydration. The presence of relatively high yields of oh^{5}dC and oh^{5}dU in oxidized DNA compared to that in oxidized dC in solution suggests that deamination and dehydration are greatly enhanced in the polymer, probably because these processes restore normal base stacking. The formation of dUg and oh^{5}dC from dC oxidation in solution make up about 40% of the total products (J.R.W., unpublished results). In contrast, the combined *G* value for the formation of dUg, oh^{5}dC, and oh^{5}dU is 0.082 (Table 1) and corresponds to 3.3% of the total DNA damage or 13% of dC damage occurring in DNA by ·OH, assuming that the maximum *G* value of DNA damage is 2.5 and that dC damage is 25% of this (42). Therefore, the formation of dCg is a major pathway of dC oxidation in DNA by OH radicals, but this pathway is suppressed 3-fold compared to that of dC in solution.

We have previously calculated on the basis of oxo^{8}dG that the DNA of each rat cell has about 10^6 oxidative adducts (4). The presence of oh^{5}dC and dUg in rat DNA supports these previous findings and also indicates that cellular DNA damage by endogenous oxygen species is extensive. The steady-state levels of oh^{5}dC and dUg in rat liver, kidney, and brain DNA are similar to those of oxo^{8}dG (Table 2) (4). Rat brain DNA contained significantly more oh^{5}dC and dUg, suggesting that oxidative damage to this organ is more extensive or is not repaired as efficiently as in liver or kidney. A higher steady-state level of these products in total brain DNA may be related to a higher rate of O_2 utilization, a higher oxidizable lipid content, and a lower level of some antioxidants in brain compared to other organs (43). The existence of oxo^{8}dG as well as oh^{5}dC and dUg in the genome implies that these lesions are not completely repaired by the organism, and thus they may contribute to mutagenesis. The mutagenesis of oxo^{8}dG has been studied in detail (27, 44–46). The oxo^{8}dG lesion has been shown to induce misreading during DNA synthesis and lead to point mutations at specific oxo^{8}dG loci in *Escherichia coli*. A molecular study of oligonucleotides containing oxo^{8}dG has demonstrated that oxo^{8}dG mispairs with dA by adopting a syn conformation, thereby implying G·C → T·A transversions in mutagenesis. The high steady-state level of oxo^{8}dG in the genome may reflect the inability of specific glycosylases to completely repair these lesions. Last, a number of studies implicate oxo^{8}dG lesions and G·C → T·A transversions in carcinogenesis (47).

The mutagenicity and repair of dC oxidation products has not been established. The presence of endogenous oh^{5}dC and dUg in cellular DNA suggests that these lesions, similar to oxo^{8}dG, are not fully repaired. dC oxidative lesions may be repaired by broad spectrum deoxyendonucleases, which recognize and excise pyrimidine oxidation products. *E. coli* endonuclease III has been shown to excise thymine glycol, 5-hydroxy-5-methylhydantoin, and hydroxymethyluracil from oxidized DNA (48). The specificity of *E. coli* endonuclease III can probably be extended to include the bulk of dC oxidation products since it introduces nicks not only at dT but also dC damaged sites and with about equal efficiency (49). Alternatively, the repair of oh^{5}dC and oh^{5}dU is likely accomplished by other repair systems, possibly similar or identical to the repair of dU, 5-hydroxymethyl-2′-deoxyuridine, and 5-hydroxymethyl-2′-deoxycytidine by specific glycosylases (26, 50, 51).

The mutagenesis of dC oxidative lesions is implicated in the formation of G·C → A·T transitions. Although both G·C and A·T base pairs are damaged by reactive oxygen species with about equal efficiency, a number of recent studies

suggest that G·C damage is more likely to lead to base changes of which the majority are G·C → A·T transitions. The spectrum of spontaneous mutations in the *lacI* gene of *E. coli* and in the SupF gene of transfected human cells is nonrandom, with 50% or more of base changes represented by G·C → A·T transitions (52, 53). In addition, the same tendency toward G·C → A·T transitions holds for ionizing radiation, H_2O_2, and Cu^+ induced mutations (53–55). The mechanism of G·C → A·T transitions likely involves oxidation of dC, which induces deamination (i.e., dC → dUg with a consequent change in the base pairing from dG to dA). In addition, DNA polymerases are strongly biased toward the incorporation of dA opposite noninstructional lesions and apurinic sites, which may arise by spontaneous or enzyme-mediated release of modified bases (56). Damage to dC residues appears to be the principal cause of G·C → A·T transitions, since damage to dG leads to G·C → T·A transversions by the above pathways as well as by the formation of oxo^{8}dG, which is a major oxidative lesion of dG. The mutagenesis of dUg lesions can thus result from deamination of dC and mispairing with dA, leading to G·C → A·T transitions. It is surprising that the steady-state level of this lesion is so high compared to dTg (estimated to be <0.5 μg of DNA; ref. 22), which has been shown to be weakly mutagenic (0.2–0.4%; ref. 57). One explanation for this is that the primary lesion may be dCg, which is sufficiently stabilized to allow its accumulation in cellular DNA. The mutagenesis of oh^{5}dU may also be caused by faulty pairing with dA. The steady state of this lesion is relatively low (<0.5 fmol/μg of DNA) compared to dUg and oh^{5}dC, although it is formed to an equal extent by OH-induced oxidation of DNA. This suggests efficient repair of this lesion in cellular DNA. In contrast to dUg and oh^{5}dU, oxidation of dC into oh^{5}dC is not likely to interfere with its base pairing with dG. On the other hand, oh^{5}dC resembles 5-methyl-2′-deoxycytidine, which is involved in gene inactivation during differentiation. Thus, oxidative damage of dC to form oh^{5}dC could possibly switch off genes.

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