Urinary 8-hydroxy-2'-deoxyguanosine as a biological marker of *in vivo* oxidative DNA damage

(aging/cancer/reactive oxygen species/HPLC electrochemical detection)

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ABSTRACT DNA is subject to constant oxidative damage from endogenous oxidants. The oxidized DNA is continuously repaired and the oxidized bases are excreted in the urine. A simple routine analytical procedure is described for urinary 8-hydroxy-2'-deoxyguanosine, an oxidative DNA damage adduct, as an indicator of oxidative damage in humans and rodents. This adduct was purified from human urine and characterized. The described assay employs a series of solid-phase extraction steps that separate 8-hydroxy-2'-deoxyguanosine from other urinary constituents, followed by analysis by gradient reversed-phase HPLC coupled to a dual-electrode high-efficiency electrochemical detection system. Analysis of urine from three species by this method indicates that mice excrete approximately 3.3-fold more 8-hydroxy-2'-deoxyguanosine than humans (582 vs. 178 residues per cell per day), a result that supports the proposal that oxidative damage to DNA increases in proportion to species-specific basal metabolic rates.

Biological damage caused by reactive oxygen species such as O_2^- , H_2O_2 , $\cdot OH$, and 1O_2 (1, 2) has been proposed to contribute to aging as well as a number of degenerative processes associated with aging such as cancer, heart disease, and cataract formation (3, 4). Various methods for detecting damage caused by these reactive oxygen species *in vitro* have led to the identification of oxidatively modified DNA bases (5).

To study the relationship between endogenous oxidative damage to DNA and degenerative diseases associated with aging, it is desirable to develop techniques that can be used for the routine quantitative analysis of these damage products in individuals. Analytical approaches such as GC/MS and immunochemical techniques have been shown to be effective methods for detecting a number of specific lesions caused by reactive oxygen species, including the modified bases 8-hydroxyguanine (oh^8Gua), 8-hydroxyadenine, and thymine glycol (5, 6). In general, these methods are useful for detecting the effects of high levels of damage caused by irradiation but are less useful for measuring levels of oxidative damage generated by endogenous metabolic processes. Previous efforts aimed at quantitating endogenously produced oxidative DNA damage involved measuring the urinary levels of thymine glycol and thymidine glycol (7, 8) by HPLC combined with UV detection. However, this method also has certain limitations, principally the time and difficulty required to perform the assay.

The inherent problem associated with detecting low levels of oxidatively damaged bases of DNA has been resolved by the identification of an oxidized DNA adduct, 8-hydroxy-2'-deoxyguanosine (oh^8dG) (9), which can be quantitated with a high degree of sensitivity and selectivity by electrochemical detection (EC) after separation of enzymatic hy-

drolyzates of DNA by reversed-phase HPLC (HPLC/EC) (10). With this sensitive and selective technique, endogenous levels of oh^8dG , which are increased upon exposure to oxidative conditions, have been measured in DNA isolated from various sources including mouse liver (11), HeLa cells (11), and rat kidney (12). In addition, oh^8dG has been shown to be present in mitochondrial DNA at a level ≈ 16 -fold higher than that present in nuclear DNA, an observation consistent with the high levels of reactive oxygen species generated in this organelle (13).

The availability of this sensitive marker of oxidative DNA damage and our overall interest in the role this damage may play in aging and cancer (3, 7, 8) has prompted the development of a noninvasive assay that can detect this damage using the electrochemical technique described above. Due to the complex nature of urine, our efforts were aimed at unambiguously confirming the existence of urinary oh^8dG and designing experiments to eliminate the possibility of artifacts. In the present report, we describe the routes that were taken to purify and characterize oh^8dG from human urine. Application of this assay to urine samples obtained from humans and rodents provides an example of its utility for the *in vivo* measurement of oxidative damage to DNA.

MATERIALS AND METHODS

Animals and Urine Collection. Mice were housed in metabolic cages and fed mouse chow ad libitum. Urine (24-hr pool) was collected under mineral oil and daily voids were stored at $-20^\circ C$. Samples were collected over 3 consecutive days and pooled. Aliquots of 1 ml were assayed. Fischer 344 rats ($n = 6$) (2–4 months of age) were obtained from Harlan-Sprague Dawley (Indianapolis, IN), housed in metabolic cages for 3–4 days prior to collection of urine samples, and fed ad libitum. Emory mouse ($n = 11$) (12 months of age) urine was kindly provided by Alan Taylor, Tufts University (Boston, MA). Human urine was obtained from normal subjects ($n = 8$) (20–60 years of age).

Synthesis of Standards. Nonradiolabeled oh^8dG standard was synthesized as described (14). 8-[1',2'- 3H]Hydroxy-2'-deoxyguanosine ($[^3H]oh^8dG$) was synthesized using a modification of this protocol and employed the following steps: 2'-[1',2'- 3H]deoxyguanosine ($[^3H]dG$) (250 μCi , 49 Ci/mmol, 5.1 nmol; 1 Ci = 37 GBq; Amersham) was transferred to a 1-ml-capacity Reacti-Vial (Pierce) and concentrated under a stream of argon to remove ethanol. $[^3H]dG$ was then purified by HPLC on a 5- μm Supelcosil LC-18 HPLC column (25 cm $\times$ 4.6 mm) (Supelco) with 10% (vol/vol) MeOH in water at a flow rate of 1 ml/min (retention time = 10 min) and concentrated to dryness prior to the hydroxylation reaction. Hydroxylation of $[^3H]dG$ at the C-8 position

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Abbreviations: EC, electrochemical detection; SPE, solid-phase extraction; oh^8dG , 8-hydroxy-2'-deoxyguanosine; dG, 2'-deoxyguanosine; oh^8Gua , 8-hydroxyguanine.

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was achieved by adding, in the following order, 71 μ l of freshly prepared 1.7 M ascorbic acid, 2 μ l of 0.2 M CuSO_4 , and 145 μ l of 31% (vol/vol) H_2O_2 to 5.1 nmol of [^3H]dG in 30 μ l of water. The extremely vigorous reaction was complete in 20 sec. The [^3H]oh 8 dG was purified immediately by HPLC as above (mobile phase = 5% MeOH in water at a flow rate of 1.7 ml/min; retention time = 16.5 min). The [^3H]oh 8 dG was collected (yield 15%), stored as is in the HPLC solvent at 4°C, and used directly. oh 8 Gua, 2-amino-6,8-dihydroxy-purine, was purchased from Chemical Dynamics (South Plainfield, NJ).

Purification and Characterization of oh 8 dG from Human Urine. Human urine (500 ml) pooled from nine healthy volunteers was made radioactive with 160,000 cpm (7.4 pmol) of a [^3H]oh 8 dG standard and passed under vacuum through a 0.2- μ m pore-size Nalgene filtration apparatus. The filtrate was divided equally into three fractions and applied to three separate 10-g C_{18} MegaBond Elut columns (Analytichem, Harbor City, CA) preconditioned with 60 ml of MeOH, 60 ml of H_2O , and 60 ml of 50 mM KH_2PO_4 (pH 7.5, buffer A). Each column was washed with 50 ml of buffer A and oh 8 dG was eluted from these columns with 50 ml of 15% MeOH/buffer A. Pooled eluants were concentrated *in vacuo* to $\approx$ 20 ml and applied to a fourth C_{18} MegaBond Elut column conditioned as above. Vacuum was applied and the column was washed with 20 ml of water. The column was dried under vacuum for 10–15 min and adsorbed material was eluted with 20 ml of MeOH. The sample was concentrated under argon to 10 ml and applied to a 10-g silica MegaBond Elut column that had been conditioned with CH_2Cl_2 . The silica column was washed with 120 ml of acetonitrile (CH_3CN) followed by 40-ml washes of 5% and 10% MeOH in CH_3CN . oh 8 dG was eluted with 60 ml of 20% MeOH in CH_3CN and concentrated to dryness. The dried residue was resuspended in 2 ml of H_2O , filtered through a 0.2- μ m pore-size nylon filter, and purified by HPLC. The initial step of HPLC purification of oh 8 dG employed a semipreparative Supelco 5- μ m Supelcosil LC-18 HPLC column (25 cm $\times$ 10 mm), at a flow rate of 2 ml/min using a mobile phase consisting of 10% MeOH in H_2O . Radioactivity that was detected in fractions corresponding to the elution volume of oh 8 dG were collected, pooled, and concentrated *in vacuo*. The previous fraction was concentrated to dryness, resuspended in H_2O , and purified by strong cation-exchange HPLC. For this step, a Supelco 5- μ m Supelcosil LC-SCX column (25 cm $\times$ 4.6 mm) and a mobile phase of 10 mM KH_2PO_4 (pH 3.5) at a flow rate of 1 ml/min were used. The radioactive fraction eluting at a retention time of 8.1 min was collected and concentrated. This fraction was separated further on an LC-18 column (25 cm $\times$ 4.6 mm) using a linear 0–10% (vol/vol) CH_3CN gradient over 50 min at a flow rate of 1 ml/min. For this analysis the HPLC effluent was monitored at 293 nm and peaks were scanned between 210 and 350 nm with the aid of an on-line Hewlett–Packard model 1040A diode array detector. The peak eluting at a retention time of 34.9 min exhibited a UV spectrum identical to that of authentic oh 8 dG. oh 8 dG was characterized by hydrolysis to the corresponding base oh 8 Gua under thermal and acidic conditions. Approximately 5 μ l of 1 M HCl was added to 20–30 pmol (in 50 μ l of water) of the putative nucleoside purified from human urine or synthetic oh 8 dG and heated for 1 hr at 90°C in separate 0.5-ml Reacti-Vial containers sealed with Pierce Tuf-Bond Teflon/silicone-lined caps. After this reaction, the mixture was concentrated to dryness and resuspended in 50 μ l of water, and a 10- μ l aliquot was analyzed by HPLC/EC at a potential of 0.6 V vs. an Ag/AgCl reference electrode. For this determination a model BAS LC-4B electrochemical detector (West Lafayette, IN) equipped with a glassy carbon working electrode was utilized.

Analytical Sample Preparation and Analysis: Solid-Phase Extraction (SPE) of Urine. Urine samples were stored at -20°C prior to workup and analysis. To 1–5 ml of urine, an equal volume of 1 M NaCl and $\approx$ 20,000 cpm of [^3H]oh 8 dG were added. The mixture was vortex mixed and applied to a 500-mg Bond Elut LRC C_{18}/OH SPE column (Analytichem) preconditioned with 10 ml of MeOH, 10 ml of water, and 10 ml of buffer A. The SPE columns were arranged on a vacuum manifold and elution of solvent washes and eluants containing the analyte was effected by applying a slight vacuum. All SPE column washes described subsequently were discarded. The column was washed in succession with 4 ml of buffer A and 5 ml of buffer B (5% MeOH in buffer A). To elute oh 8 dG, 3 ml of buffer C (15% MeOH in buffer A) was applied to the column. This eluant was transferred to a second C_{18} -OH column preconditioned as described above. The eluant from the previous column was applied and then an additional 1 ml of water was added to remove most of the remaining buffer salts. The column was dried thoroughly under vacuum for 10–15 min and then oh 8 dG was eluted from this column with 1 ml of MeOH. The eluant containing oh 8 dG was placed in a water bath at 35–40°C and concentrated to dryness under a gentle stream of argon. The sample was resuspended in 100 μ l of MeOH and applied to a 500-mg Bond Elut silica SPE column preconditioned with 3 ml of CH_2Cl_2 . This column was washed in succession with 6 ml of CH_3CN , 2 ml of 5% MeOH in CH_3CN , 2 ml of 10% MeOH in CH_3CN , and 1 ml of 20% MeOH in CH_3CN . oh 8 dG was then eluted from this column with a further 3-ml aliquot of 20% MeOH in CH_3CN . The eluant was concentrated to dryness and the white residue was resuspended in 500 μ l of HPLC-grade water. From the 500- μ l sample, 25–100 μ l was analyzed by HPLC/EC. The amount of radiolabeled tracer present in the remaining sample was quantitated by liquid scintillation counting to determine the recovery ($\approx$ 35% in most cases).

HPLC/EC Analysis of Urinary oh 8 dG. Waters models 510 and 6000A solvent delivery systems equipped with a Waters model 710B WISP autoinjector were used. Separation of oh 8 dG by HPLC employed a Supelco 5- μ m Supelcosil LC-18-S precolumn cartridge assembly (2 cm $\times$ 4.6 mm) linked to two 5- μ m Supelcosil LC-18-S analytical columns (25 cm $\times$ 4.6 mm) attached in series. oh 8 dG was eluted at a flow rate of 1 ml/min. The solvents used for gradient elution are as follows: solvent A (50 mM KH_2PO_4 at pH 5.5) and solvent B [50% $\text{CH}_3\text{CN}/\text{MeOH}$, 7:3 (vol/vol), in solvent A]. The conditions for gradient elution are as follows: 0–80 min, 0–8% solvent B; 80–85 min, 8% solvent B isocratic; 85–90 min, 8–50% solvent B; 90–100 min, 50% solvent B isocratic; 100–105 min, 50–0% solvent B. In all cases, a linear ramp was used when solvent compositions were changed. Before proceeding with the analysis of each subsequent sample, a 45-min equilibration at the initial conditions was programmed. Urinary oh 8 dG was detected by an ESA model 5100 Coulochem detector equipped with a 5011 high-sensitivity analytical cell. The potentials for electrodes 1 and 2 were adjusted to 0.12 and 0.40 V, respectively. A preinjector guard cell set at a potential of 0.40 V was also employed. The detection was normally performed at 200 nA, full scale (gain = 500) and reprocessed according to the signal obtained for oh 8 dG. A Knauer dynamic mobile-phase mixer plus two-pulse dampers (SSI, State College, PA) were attached in series to minimize the fluctuations in baseline signal current. Data were digitized by a Nelson 760 (Cupertino, CA) analytical interface and processed by Nelson analytical series 4400 data acquisition software on a Hewlett–Packard 9816 computer.

[^3H]oh 8 dG and [^3H]dG and their products of chemical or enzymatic decomposition were separated by HPLC with an LC-18 analytical column (25 cm $\times$ 4.6 mm) using a mobile phase containing 10% MeOH in water and detected by a

radiochemical flow detector (model Flo-one/Beta, Radio-matic Instruments and Chemical, Tampa, FL). Flow rates for HPLC and ScintiVerse LC scintillation fluid (Fisher Scientific) were 1 and 2.5 ml/min, respectively. The radioactivity flow detector employed a 0.5-ml flow cell.

RESULTS

Purification and Characterization of oh^8dG from Normal Human Urine. To identify oh^8dG as a constituent of human urine unambiguously, preparative purification was performed. Human urine pooled from nine healthy individuals was purified by a series of preparative SPE and HPLC steps, and the methods described below were used to characterize putative oh^8dG . As shown in Fig. 1, putative oh^8dG purified by SPE and HPLC from human urine eluted at 34.8 min. This peak, which comigrated with $[\text{H}^3]\text{oh}^8\text{dG}$ added prior to purification, displayed absorption maxima at 245 and 293 nm, as determined by UV diode array spectral analysis (Fig. 1 *Inset*). UV spectra taken at the upward and downward inflections as well as the apex of this peak were superimposable, indicating that the peak corresponding to putative oh^8dG was comprised of a single UV-absorbing component. Although oh^8Gua and 8-hydroxyguanosine also exhibit identical UV spectra, these compounds elute several minutes earlier than oh^8dG when analyzed under these conditions. In addition hydrodynamic voltammogram measurements of this compound showed that the electrochemical properties ($E_{1/2} = 0.46 \text{ V vs. Ag/AgCl}$) were identical to that of synthetic oh^8dG (data not shown). Additional characterization of putative urinary oh^8dG was provided by assessing the acid and thermal hydrolysis product of this compound. A portion of the purified sample described above was heated at 90°C for 1 hr in 0.1 M HCl, conditions that hydrolyze the N-glycosidic bond of oh^8dG . This treatment produced a quantitative shift in the elution time of putative oh^8dG from 11.2 min to 5.0 min as detected by HPLC/EC (Fig. 2, traces A and B). Treatment of synthetic oh^8dG in the same manner also produced this quantitative shift in peak retention times (Fig. 2, traces C and D). The acid and thermal degradation product derived from the biological isolate and synthetic oh^8dG migrated with a retention time identical to oh^8Gua , the anticipated hydrolysis product (Fig. 2, trace E). To confirm this assignment, synthetic oh^8Gua was analyzed and found to comigrate with the hydrolyzate obtained from the biological and synthetic samples (Fig. 2, trace F).

HPLC/EC of Urinary oh^8dG . Fig. 3 shows a typical chromatogram produced from a SPE-processed sample of normal human urine. Under the conditions described, oh^8dG elutes at 81.8 min as a well-resolved peak. Fig. 3 *Inset* shows a partial chromatogram of the same sample to which 2.5 pmol of synthetic oh^8dG had been added. The value of oh^8dG obtained by this analysis was normalized to the volume of urine excreted in a 24-hr period and the body weight of the

FIG. 1. Diode array UV spectral analysis of putative human urinary oh^8dG . SPE- and HPLC-processed pooled human urine was analyzed by HPLC at 293 nm with diode array UV detection. (*Inset*) Diode-array UV spectrum of putative human urinary oh^8dG (—) and synthetic oh^8dG (---). UV spectra have been offset for clarity. mAU, absorption units ($\times 10^{-3}$).

FIG. 2. Acid- and thermal-catalyzed hydrolysis of putative human urinary oh^8dG and synthetic oh^8dG . A portion of the biological isolate ($\approx 3 \text{ pmol}$) and synthetic oh^8dG (5 pmol) was heated for 1 hr at 90°C in 0.1 M HCl. These mixtures, after a concentration and resuspension step, were analyzed by HPLC/EC ($0.6 \text{ V vs. Ag/AgCl}$) and compared to unhydrolyzed samples and synthetic oh^8Gua . Traces A and B show HPLC/EC chromatograms of a biological isolate ($\approx 2 \text{ pmol}$) before hydrolysis and after hydrolysis ($\approx 4 \text{ pmol}$), respectively. Traces C and D show HPLC/EC chromatograms of synthetic oh^8dG before and after hydrolysis, respectively ($\approx 5 \text{ pmol}$ injected on column for each analysis). Trace E shows the coelution of 2.5 pmol of synthetic oh^8Gua and $\approx 2.5 \text{ pmol}$ of biological isolate after hydrolysis. Trace F shows 5 pmol of synthetic oh^8Gua .

individual. To confirm the accuracy of this quantitative measurement, levels of oh^8dG in urine samples processed by SPE ($323 \pm 23 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, $n = 5$) or purified exhaustively to homogeneity by HPLC ($294 \pm 15 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, $n = 4$) were measured and judged to be identical.

Urinary oh^8dG Does Not Appear to Be an Artifact of Enzymatic or Chemical Oxidation Processes. Urine contains measurable amounts of ascorbic acid and transition metals such as copper (15, 16), components that, in the presence of peroxides, catalyze the hydroxylation of 2'-deoxyguanosine (dG) to oh^8dG . It is conceivable, therefore, that trace quantities of dG that may be present in urine could be transformed chemically to oh^8dG prior to analysis. To assess this possibility $[\text{H}^3]\text{dG}$ was injected intravenously into a rat tail vein and urine was collected over the subsequent 24-hr period. Approximately 4.9% of the radioactivity was recovered in the first 24-hr collection with insignificant amounts recovered on subsequent days, suggesting that most of the $[\text{H}^3]\text{dG}$ was incorporated into cellular nucleoside pools. Radiochemical

FIG. 3. Gradient reversed-phase HPLC/EC chromatogram of SPE-processed human urine. The analysis was performed using a screening potential of 0.12 V (electrode 1, coulometric) and an electrode potential of 0.40 V (electrode 2, amperometric). (*Inset*) A partial EC chromatogram of the same sample with 2.5 pmol of authentic oh^8dG added. Approximately 650 fmol of oh^8dG is detected from the equivalent of 59 μl of normal human urine. The contribution of the radiolabeled standard (7.2 fmol) is negligible, accounting for $\approx 1\%$ of the total signal obtained from this sample.

analysis of the first 24-hr urine sample indicated that dG was degraded by $\approx 70\%$; however, no oh^8dG was detected. Furthermore, no oh^8dG was produced when $[\text{H}]^3\text{dG}$ was stored in urine for 19 days at 4°C , indicating that the chemical transformation of dG to oh^8dG by urinary constituents is not significant.

Various purine derivatives have been shown to be converted by cytosolic enzymes such as aldehyde oxidase to the corresponding C-8 hydroxylated purines (17). To determine whether urinary oh^8dG could potentially be derived from enzymatic oxidation of free cytosolic pools of dG, $10\ \mu\text{M}$ $[\text{H}]^3\text{dG}$ was incubated for 60 min at 37°C with the $100,000 \times g$ soluble fraction (1 mg/ml) isolated from rat liver. Although $[\text{H}]^3\text{dG}$ was converted rapidly to at least three chromatographically distinct peaks, measured after HPLC separation by radiochemical detection, $[\text{H}]^3\text{oh}^8\text{dG}$ could not be detected, indicating that soluble enzymes lack the activity necessary to convert this nucleoside to oh^8dG .

Another potential source of C-8 hydroxylase activity is the microsomal cytochrome P-450 system. Incubation of $10\ \mu\text{M}$ $[\text{H}]^3\text{dG}$ at 37°C for 1 hr with microsomes (1 mg/ml) isolated from mouse liver in the presence or absence of an NADPH-regenerating system failed to produce oh^8dG . Although dG was metabolized to an extent of $\approx 30\%$ in a process that appeared to be independent of NADPH, no measurable levels of oh^8dG could be detected. Therefore, it is unlikely that urinary pools of oh^8dG are derived from cytochrome P-450 catalyzed oxidation of dG.

Administration of $[\text{H}]^3\text{oh}^8\text{dG}$ to rats by gastric intubation was performed to test whether dietary sources of oh^8dG might contribute to the level detected by our assay. After 2 μCi of $[\text{H}]^3\text{oh}^8\text{dG}$ was administered, 24-hr urine samples were collected over the next 7 days. $[\text{H}]^3\text{oh}^8\text{dG}$ and its radiolabeled decomposition products were detected by radiochemical detection after separation of urine by HPLC. Radiochemical analysis of these samples indicated that only the first 24-hr urine sample contained intact $[\text{H}]^3\text{oh}^8\text{dG}$ in an amount corresponding to 1% of the administered dose. Although radioactivity was recovered over the subsequent 6 days of collection, only early eluting decomposition products were observed. We estimate from the relative amount of $[\text{H}]^3\text{oh}^8\text{dG}$ recovered that the contribution of dietary oh^8dG represents $<2\%$ of the total oh^8dG detected by our assay.

To validate the utility of oh^8dG as a marker of oxidative stress, the stability of this compound was tested after intravenous injection and subsequent collection of urine. Results of this experiment show that of 4 μCi of $[\text{H}]^3\text{oh}^8\text{dG}$ injected intravenously into the tail vein of a rat 66% is recovered in the first 24-hr collection. HPLC separation of this urine sample followed by radiochemical analysis did not reveal any measurable degradation of oh^8dG after administration and excretion.

oh^8dG Levels in Humans and Rodents. To test the proposal that urinary excretion of oxidatively damaged products of DNA is related to species-specific metabolic rate, the levels of oh^8dG present in 24-hr urine samples obtained from human, rat, and mouse were measured. Urinary output of oh^8dG , measured by HPLC/EC following SPE processing, was plotted as a function of estimates for species-specific O_2 consumption (Fig. 4). The correlation between urinary excretion of oh^8dG and O_2 consumption was high among the three species examined ($R = 0.98$). The difference between mouse and human oh^8dG values was ≈ 3.3 -fold.

DISCUSSION

Reactive oxygen species, which are produced continuously as a consequence of normal metabolism, have been proposed to cause damage to critical biomacromolecules such as DNA (18). This damage, if left unrepaired, may contribute to aging

FIG. 4. Comparison of urinary oh^8dG levels among three species. oh^8dG levels (mean $\pm$ SEM) from human, rat, and mouse vs. species-specific metabolic rates. Numbers of samples analyzed are shown in parentheses.

and degenerative diseases associated with it (7, 8, 18). The identification in DNA of oh^8dG , one oxidative DNA damage product of about 20 described (19), has led to a number of studies aimed at understanding the relationship between oxidative DNA damage and age-associated degenerative processes. Although the consequence of this damage *in vivo* remains unknown, studies utilizing DNA templates containing oh^8dG indicate that this oxidatively modified residue causes mispairing, suggesting that this lesion may be mutagenic (20).

The assay described in the present report is an extension of previous efforts aimed at assessing the body load of oxidative DNA damage. Our reasons for developing the present method were prompted by the poor sensitivity and the long sample workup time required for the measurement of urinary thymine glycol and thymidine glycol (7, 8). The noninvasive assay offers, in addition to the advantages described for the measurement of these oxidative adducts, ≈ 100 -fold greater sensitivity and more rapid sample processing (2 days vs. 2 weeks for 12 samples) than the method described previously (7, 8).

We have addressed some of the possible routes of artifactual formation of oh^8dG . Enzymatic and chemical oxidation of dG to oh^8dG is unlikely, since cytosolic enzymes and cytochrome P-450 containing microsomal preparations do not catalyze the oxidation of this compound. In addition, dG does not appear to be chemically transformed to oh^8dG and excreted when injected intravenously, nor does similarly administered oh^8dG degrade prior to its excretion into urine. Dietary oh^8dG also does not appear to contribute significantly to the urinary oh^8dG detected by this assay. The chemical stability of oh^8dG also exhibited favorable properties, as judged by its stability after storage in urine for an extended period.

In the present study we have established the presence of oh^8dG , a product of oxidative damage to DNA, in urine obtained from human subjects. A procedure based on SPE followed by HPLC/EC is described for the routine quantitative analysis of oh^8dG in urine obtained from humans, rats, and mice. The total amount of oh^8dG excreted by humans was estimated to be $130\text{--}300\ \text{pmol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, a value that is similar to the level of urinary thymidine glycol ($100\ \text{pmol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) reported (7). We estimate, using the approach of Cathcart *et al.* (7), that a normal human individual excretes 5.1×10^{15} residues of oh^8dG per day. This value corresponds to an average of 168 residues of oh^8dG per cell per

day.[†] Therefore, as in the case of thymidine glycol, dG is damaged oxidatively, repaired, and excreted into the urine.

oh⁸dG is detected also in urine samples obtained from rats and mice. The weight-corrected level of urinary oh⁸dG is ≈1.5- and 3.3-fold greater than that detected in human urine. The levels of urinary oh⁸dG in each species appear to correlate well with species-specific metabolic rate, as was shown for thymidine glycol (8), and are consistent with the inverse correlation between species-specific metabolic rate and longevity (21). The differences between human and rat and between human and mouse oh⁸dG levels, however, are not as pronounced as for thymidine glycol, where 15- and 26-fold differences, respectively, were observed (8). The reason for this marked difference is unknown. Differences in the activity of repair enzymes of oxidatively modified lesions present in DNA could alter dramatically the relative amount of the nucleoside detected in urine. The ratio of the activities that release oh⁸dG to those that release oh⁸Gua in the three species studied thus would influence the relative differences observed for each of these two oxidatively modified compounds. Since it has not yet been possible to accurately determine the urinary levels of the corresponding base, oh⁸Gua, it is not yet possible to make this assessment though this base is present in both rat (22) and human urine (M.K.S. and B.N.A., unpublished data).

Our findings are consistent with the proposal that the steady-state level of oxidative damage to DNA may be an important determinant of species-specific life span. Application of this noninvasive assay may be useful for examining various conditions associated with aging and cancer.

[†]The value of 168 oh⁸dG residues per cell per day was calculated based on the assumption that a human contains 5×10^{13} cells per 70 kg (7.14×10^{11} cells per kg); 200 pmol of oh⁸dG per kg per day is equivalent to 1.2×10^{14} residues per kg per day; when this value is divided by 7.14×10^{11} cells per kg, the result is equivalent to 168 oh⁸dG residues per cell per day.

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