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DNA oxidation matters: The HPLC–electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine

(adducts/aging/cancer/mutation/oxidants)

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ABSTRACT **Oxidative DNA damage is important in aging and the degenerative diseases of aging such as cancer. Estimates commonly rely on measurements of 8-oxo-2′-deoxyguanosine (oxo^{8}dG), an adduct that occurs in DNA and is also excreted in urine after DNA repair. Here we examine difficulties inherent in the analysis of oxo^{8}dG, identify sources of artifacts, and provide solutions to some of the common methodological problems. A frequent criticism has been that phenol in DNA extraction solutions artificially increases the measured level of oxo^{8}dG. We found that phenol extraction of DNA contributes a real but minor increase in the level of oxo^{8}dG when compared, under equivalent conditions, with a successful nonphenol method. A more significant reduction in the baseline level was achieved with a modification of the recently introduced chaotropic NaI method, reducing our estimate of the level of steady-state oxidative adducts by an order of magnitude to 24,000 adducts per cell in young rats and 66,000 adducts per cell in old rats. Of several alternative methods tested, the use of this chaotropic technique of DNA isolation by using NaI produced the lowest and least variable oxo^{8}dG values. In further studies we show that human urinary 8-oxo-guanine (oxo^{8}Gua) excretion is not affected by the administration of allopurinol, suggesting that, unlike some methylated adducts, oxo^{8}Gua is not derived enzymatically from xanthine oxidase. Lastly, we discuss remaining uncertainties inherent both in steady-state oxo^{8}dG measurements and in estimates of endogenous oxidation ("hit rates") based on urinary excretion of oxo^{8}dG and oxo^{8}Gua.**

Oxidants from metabolic activity, inflammation, radiation, or toxins can damage nucleic acids, generating lesions that appear to contribute to aging and cancer (1–7). About 20 major oxidative DNA adducts have been characterized (8). One of these is 8-oxo-2′-deoxyguanosine (oxo^{8}dG), an adduct for which specific cellular repair enzymes exist and that has been shown to cause G-to-T transversions (9). Although there is little doubt that oxo^{8}dG is an endogenous mutagenic lesion, numerous quantitative questions remain: just how much oxo^{8}dG is there *in vivo*? What are the rates of its formation and repair? Does it accumulate with age, and if so, is this because of increased formation or decreased repair? Intriguing as these questions are, finding definitive answers has been hampered by technical obstacles and physiological unknowns.

Oxo^{8}dG and its corresponding base 8-oxo-guanine (oxo^{8}Gua) have proved particularly useful because of the selectivity and sensitivity with which they can be quantified by electrochemical (EC) detection (10). Initially, HPLC-EC was used to quantify oxo^{8}dG in DNA hydrolysates as a measure of the steady-state level of DNA oxidation *in situ*. Later, the development of a monoclonal antibody specific for oxo^{8}dG allowed purification of the adducts directly from urine, blood, and tissue culture medium (11), leading to the hope that urinary measurements would represent an integrative measure of endogenous damage.

The dissemination of oxo^{8}dG and oxo^{8}Gua methods has resulted in a burgeoning literature on oxidative DNA damage (12). At the same time, however, modifications and alternatives to the initial HPLC-EC techniques have been introduced, leading to discordant results, the identification of various pitfalls, and disagreements about the most appropriate technique (13–23). Because of the broad interest in using oxo^{8}dG as a biomarker, it is critical to identify a common set of methods that minimize error in measurement of the adduct. Here, we have evaluated various published methods for isolating DNA and processing samples and have investigated sources of artifacts. Our studies include (*i*) the use of a new chaotropic technique (19), (*ii*) the use of phenol, (*iii*) the analysis of very small quantities of DNA ($\leq$20 μg), (*iv*) the duration of DNA hydrolysis, (*v*) the presence of redox-active metals, (*vi*) the conditions of DNA extraction, (*vii*) chromatographic interference, (*viii*) recovery of adducts during immunoaffinity purification, and (*ix*) the formation of oxidized guanine derivatives by xanthine oxidase (24).

In addition to technical problems there are theoretical uncertainties in the use of oxo^{8}dG as an oxidative biomarker. Therefore, we discuss the interpretation of oxo^{8}dG in the light of currently unmeasured and potentially confounding pathways of its formation and breakdown.

METHODS

Materials. Chemicals were HPLC grade where available and otherwise were reagent grade. Oxo^{8}Gua was from Fairfield Chemicals, dGMP was from Sigma, and trimethylchlorosilane (1%) in *N*,*O*-bis(trimethylsilyl)trifluoroacetamide was from Pierce. [^{14}U-C]8-oxoguanine, [1,2-^{3}H]oxo^{8}dG and oxo^{8}dG were prepared as described (25–27). WB DNA extractor kits [chaotropic NaI method (16)] were obtained from Wako Chemical (Richmond, VA).

DNA Isolation Methods. Six different DNA isolation protocols were initially compared. As a common starting material, fresh rat liver was homogenized, frozen in liquid nitrogen, and stored at −80°C until processed (<14 days). Three of these protocols were based on the recent adoption (by Nakae *et al.*, ref. 19) of chaotropic NaI as an alternative to phenol extraction and were:

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Abbreviations: oxo^{8}dG, 8-oxo-2′-deoxyguanosine; oxo^{8}Gua, 8-oxoguanine; EC, electrochemical detection; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; AP, alkaline phosphatase.

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(*i*) the chaotropic NaI method according to the manufacturer's instructions (16), (*ii*) the chaotropic method with the addition of a phenol extraction step, and (*iii*) the chaotropic method with the addition of phenol extraction and the omission of NaI (sodium acetate substituted). The fourth protocol was (*iv*) the phenol extraction procedure previously used in our laboratory (25). Two final procedures were based on prolonged (overnight) pronase incubation, as described (22, 28): (*v*) an overnight pronase digestion, and (*vi*) an overnight pronase digestion with the addition of a phenol extraction step. In all six procedures the DNA was dried by centrifugation under vacuum before digestion and the samples were analyzed immediately. DNA hydrolysis and HPLC analysis were performed as described (25) but with a desferal concentration of 0.1 mM.

Modification of the Chaotropic NaI Method. In a subsequent series of experiments, three modifications were made to the standard manufacturer's chaotropic method: (*a*) In the initial tissue homogenization, 0.1 mM desferal was added and Triton X-100 was removed from the homogenization buffer (0.32 M sucrose/5 mM $MgCl_2$/10 mM Tris·HCl, pH 7.5). (*b*) The protocol was carried out in three distinct stages (with samples stored at −80°C between stages): (*i*) tissue homogenization and collection of the nuclear pellet, (*ii*) isolation of DNA with chaotropic NaI, and (*iii*) hydrolysis of the DNA and HPLC analysis. (*c*) During stage *ii*, after the final wash of the manufacturer's protocol, DNA pellets were not vacuum dried but instead were stored at −80°C (until the third stage of the protocol). The DNA pellets were then rinsed with 20 mM sodium acetate buffer (pH 4.8), the buffer was decanted, the tubes were drained onto filter paper, fresh 20 mM sodium acetate buffer was added, and DNA hydrolysis was begun.

Analyte from Urine Samples. Human urine samples were stored at −20°C and analyzed as described (24) with the following modifications. Samples were thawed, adjusted to pH 6.9–7.2, and warmed in a 37°C water bath for 10 min. One-milliliter aliquots were loaded onto 500-mg, low hydrocarbon, C-18-OH solid phase extraction (SPE) columns (Varian), diluted with an equal volume of 1 M NaCl, and spiked with appropriate radiolabeled tracers (11). After a 1-ml wash with 50 mM potassium phosphate buffer, pH 7.5, the analyte was eluted with 15% methanol in 50 mM potassium phosphate buffer, pH 7.5.

Allopurinol Study. The effect of xanthine oxidase inhibition on urinary excretion of oxo^{8}Gua was assessed by using allopurinol, by a modification of the method of Skupp and coworkers (24). Following the collection of 24-hr urine samples for 2 control days, subjects received allopurinol for 3 days in increasing daily doses of 300, 400, and then 500 mg. Urine collections were then continued for 2 further days as postintervention control days. The study was approved by the Human Subjects Committee of the University of California, Berkeley.

Measurement Artifacts in Urinary oxo^{8}dG Measurement. When urine was processed by the immunoaffinity method, a large electrochemically active peak often was seen near the position of oxo^{8}dG elution. This peak was purified, derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide-1% trimethylchlorosilane in an equal volume of pyridine at 100°C for 30 min, and analyzed on a Hewlett–Packard 5971A GC/MS equipped with a 30 m × 0.2 mm HP-5 capillary column (0.33-mm film thickness). The column temperature was held at 60°C for 2 min, raised at a rate of 10°C min^{-1} to 250°C, and held for 3 min. Under these conditions the analyte eluted at 15.04 min.

Statistical Analysis. Mean values were compared by using a two-tailed Student's *t* test for uncorrelated means as calculated by an INSTAT program (GraphPad Software, San Diego), which was also used to generate correlation coefficients. Variation is reported as standard error of the mean.

RESULTS

The Analysis of Steady-State Levels of oxo^{8}dG in DNA from Tissues. *Comparison of DNA isolation methods and the effect of phenol.* We compared six methods of DNA extraction (see *Methods*, $n = 5$), and found that a chaotropic NaI-based method (16), recently applied to the study of oxo^{8}dG (19), resulted in the lowest values of oxo^{8}dG. Compared with the traditional phenol extraction protocol, the new NaI method resulted in a 50% lower ratio of oxo^{8}dG to dG (0.28 ± 0.04 vs. 0.54 ± 0.07 oxo^{8}dG/10^5 dG; $P < 0.02$). The addition of a phenol extraction step to the chaotropic NaI method (immediately before the addition of NaI) increased the ratio oxo^{8}dG/dG 2-fold (0.28 ± 0.04 to 0.55 ± 0.08 oxo^{8}dG/10^5 dG; $P < 0.02$), to the value equivalent to that obtained with phenol extraction alone. Although our tentative conclusion from these results was that phenol had artifactually elevated oxo^{8}dG/dG, these results could also reflect a reduction in the ratio of oxo^{8}dG to dG during the NaI step.

Consequently, to test the hypothesis that NaI in the chaotropic method had artifactually *depressed* the measured level of oxo^{8}dG (e.g., by chemical decomposition), we substituted sodium acetate for NaI in the chaotropic + phenol method and found that the value remained unchanged (0.50 ± 0.06 oxo^{8}dG/10^5 dG). Another method (22, 28), the hydrolysis of samples with pronase, increased the ratio to 1.2 ± 0.03 oxo^{8}dG/10^5 dG ($P < 0.03$) and resulted in greater variability. By using rat liver nuclei from young animals, we found that the modified chaotropic NaI procedure (see *Methods*) produced the lowest baseline values (0.04 ± 0.002 oxo^{8}dG/10^5 dG).

Analysis of microgram quantities of DNA. We previously reported that the analysis of small quantities of DNA results in higher ratios of oxo^{8}dG/dG and hypothesized that this could be the result either of (*i*) the presence of a small, coeluting peak that could disproportionately influence the integration of small oxo^{8}dG peaks, or (*ii*) the occurrence of a constant amount of artifactual oxidation, which could disproportionately influence a small quantity of DNA (29). In recent experiments we have found that interference can be caused by carryover of oxo^{8}dG from sample to sample, because of contamination of the autoanalyzer injector system, and we have eliminated the problem by meticulous attention to injector hygiene. We monitor for recurrence of the problem by including multiple blanks in the sample sequence and inspecting the resulting chromatograms for peaks eluting at the retention time of oxo^{8}dG. The effects of carryover can also be minimized by injecting larger quantities of DNA hydrolysate (e.g., ≥30 μg) to ensure that the electrochemical peak of oxo^{8}dG is significantly greater than interference from contamination.

Enzymatic hydrolysis of DNA. We digested DNA with varying amounts of hydrolytic enzymes to test the idea that oxo^{8}dG might be underestimated if it is inefficiently processed by nuclease P1 or alkaline phosphatase (AP). Increasing the amount of nuclease P1 per sample from 0.3 to 13 μg increased the DNA hydrolyzed 10 min^{-1} from 30 to 53 μg, and increased the ratio of oxo^{8}dG/10^5 dG from 0.65 to 0.90, suggesting that inefficient release of oxo^{8}dG could lead to an underestimate of about 30%. However, other experiments also suggest that nuclease P1 incubation may lead to oxo^{8}dG overestimates. The activity of nuclease P1 is optimal at 70°C, and longer incubations of DNA with the nuclease resulted in a higher ratio of oxo^{8}dG/dG. To determine whether this is a result of more complete release of oxo^{8}dG or of temperature-dependent autooxidation, the nonsubstrate dGMP was treated with nuclease P1 in control experiments. Incubation for longer than 10 min at 70°C resulted in autooxidation at the rate of 12 fmol h^{-1} nmol^{-1} dGMP, and even without incubation, the addition of nuclease P1 itself resulted in the formation of oxo^{8}dGMP (1.43 ± 0.05 vs. 1.71 ± 0.06 oxo^{8}dGMP/10^5 dGMP; $P < 0.05$).

These artifacts were reduced by limiting nuclease P1 incubation to 10 min and by adding desferal, suggesting that the artifact is a result of transition metal contamination. Studies with AP failed to demonstrate similar problems. Digestion of samples was complete after 60 min, and a constant ratio of oxo^{8}dG/dG was achieved as soon as 10 min after addition of the enzyme. We failed to observe artifactual oxidation during incubation for up to 1 hr at 37°C.

Effect of desferal. As seen in the control incubations with nuclease P1 and AP, the addition of desferal limits autooxidation. However, the inclusion of 1 mM desferal in DNA hydrolysis buffers produced artifactual peaks, baseline drift, and loss of sensitivity of the EC detectors. The use of 0.1 mM desferal eliminated these problems. The addition of desferal to the homogenization buffer produced a small but significant reduction (0.04 ± 0.002 vs. 0.06 ± 0.01; $P < 0.02$) in measured oxo^{8}dG/dG ratios. We now routinely include this chelator during homogenization.

Proteinase K digestion. To determine whether an extended proteinase K digestion would increase the lability of purified DNA to nucleases, and hence the release of oxo^{8}dG, we digested rat liver nuclear pellets overnight with proteinase K and found that the ratio of oxo^{8}dG increased roughly 2-fold (0.86 ± 0.03 vs. 0.38 ± 0.05 oxo^{8}dG/10^5 dG; $P < 0.01$). The recovery of DNA did not increase, however, suggesting that the elevated oxo^{8}dG from overnight proteinase digestion was an artifact of the extended incubation rather than the result of more efficient digestion. This interpretation is consistent with the results of the prolonged pronase exposure studies described above.

Mixing effects. In the routine phenol extraction of DNA, we have replaced the slow rocking of samples with brief vortexing. When the two methods were compared, no significant differences were found (0.45 ± 0.12 vs. 0.44 ± 0.06 oxo^{8}dG/dG).

Oxo^{8}Gua and oxo^{8}dG from Urine and Fluids. *Peak interference in urine chromatography.* The immunoaffinity purification of 8-oxoguanine adducts from urine resulted in a large HPLC-EC peak which obscured that of oxo^{8}dG. The interference worsened with column age, suggesting that it was derived from the column matrix. UV and GC/MS analysis of the interfering peak gave a good match with a disaccharide, a finding consistent with the breakdown of the column matrix. Gradient elution with a combination of methanol and acetonitrile separates the peaks and thereby resolves the problem (24).

Recovery of oxo^{8}Gua from urine. Although normal urine is clear when freshly voided, urine stored in the cold frequently develops a precipitate. Dissolving this precipitate improved and stabilized recoveries of oxo^{8}Gua. The procedure involves adjusting the pH of the samples to 6.9–7.2 and warming for 10 min at 37°C. The samples are then immediately diluted with an equal volume of 1 M NaCl and applied to SPE columns. Under these conditions, oxo^{8}Gua is retained by the SPE column but will not tolerate extensive washing. Recoveries of 60–75% were obtained when wash volumes were restricted to 1 ml. Although this method works well for oxo^{8}Gua, the limited wash volume results in numerous small peaks that obscure the oxo^{8}dG peak; urinary oxo^{8}Gua and oxo^{8}dG therefore must be analyzed separately.

Effect of allopurinol on oxo^{8}Gua excretion. Allopurinol, an inhibitor of xanthine oxidase, was administered to two healthy, adult males as described in *Methods*. Each subject contributed 2 control days and 2 postintervention control days. The effect of allopurinol was measured on the third day of drug administration. Oxo^{8}Gua excretion was not significantly different during the three time periods ($P > 0.5$).

Young vs. Old Rat Liver oxo^{8}dG Levels. By using modifications of the NaI technique (see *Methods*) we compared oxo^{8}dG levels from liver homogenates taken from young (3- to 6-month-old) vs. old (24- to 26-month-old) rats. We found that old rat liver contained 2.75 times more oxo^{8}dG than young rat liver (0.11 ± 0.01 vs. 0.04 ± 0.002 oxo^{8}dG/10^5 dG, $P < 0.01$).

DISCUSSION

The difficulty in measuring rare DNA base adducts is particularly acute in the case of oxidative damage, because artifactual oxidation of more than 0.0001% of the unmodified bases may significantly affect the outcome. At the same time, there is tremendous interest in oxidative DNA damage in aging (5, 7) and cancer (1, 3), as well as compelling genetic evidence that oxidative mutagenesis is relevant to a wide range of aerobic organisms, including humans (9). An adduct such as oxo^{8}dG should have significant potential as a biomarker (5), and during the past decade considerable effort and ingenuity have been invested in developing methods for its analysis. Here, we have compared our own and others' protocols and found that, among them, a chaotropic NaI-based technique (19) currently provides the lowest and least-variable values.

Artifacts and Obstacles in the Measurement of oxo^{8}dG in DNA. The validity of the standard HPLC-EC-based oxo^{8}dG method (25, 27, 30) often has been questioned, and modifications of the technique have been introduced (13–23). Much of the debate has surrounded the use of phenol (15, 18, 19, 22, 31) after a report (14) that phenol is a prooxidant during DNA extraction. Although we have never found phenol to cause a large increase in oxo^{8}dG, we have reinvestigated its use by adding a phenol-extraction step to phenol-free methods.

In our hands, a recently introduced chaotropic NaI extraction method (16) gives lower oxo^{8}dG/dG values than we have previously achieved with the phenol method. The addition of a phenol extraction step to this method increased the concentration of oxo^{8}dG by an amount that was modest in absolute terms (0.25 oxo^{8}dG/10^5 dG) but statistically significant. In contrast, the value we obtained with an alternative phenol-free method (22, 28) was five times higher than that obtained with the chaotropic NaI method. Moreover, this latter phenol-free value was unaffected by the addition of a phenol extraction step.

Several other laboratories have reported phenol artifacts (15, 18, 19, 22, 31), but the evidence as presented is not convincing. For instance, it has been claimed that 6-hr pronase digestion without phenol extraction results in lower oxo^{8}dG levels than phenol extraction alone, yet there was no reported difference between the two methods when applied to rat liver DNA (1.74 vs. 1.72 oxo^{8}dG/10^5 dG) (18), and the standard phenol method was in fact superior when calf thymus DNA was the starting material. Phenol-free pronase extraction was only marginally superior when butylated hydroxytoluene was included (1.13 oxo^{8}dG/10^5 dG for pronase vs. 1.25 oxo^{8}dG/10^5 dG) (18). A comparison of pronase and phenol from another laboratory is similarly hard to interpret because of the practice of drying DNA under air and results that are an order of magnitude higher than those reported here by using essentially the same method (22). Furthermore, in the initial report of the phenol artifact, the lowest values of oxo^{8}dG documented (in the absence of phenol) are far higher than we report here with or without phenol [13.7 oxo^{8}dG/10^5 dG (14) vs. 0.28–1.2 oxo^{8}dG/10^5 dG]. This discrepancy may be because of the drying of DNA under a stream of air in the initial report (ref. 14; Fig. 3), a procedure that was found to form oxo^{8}dG irrespective of prior exposure to a variety of organic solvents (15).

In support of our conclusion that the effect of phenol is minor, Kaneko *et al.* (31) were unable to demonstrate an effect of phenol on oxo^{8}dG levels, whereas Harris *et al.* (15) conclude that excessive air exposure, rather than organic extraction *per se*, is responsible for artifactual oxo^{8}dG. Finally, Nakajima *et al.* (20) report that air exposure is benign unless free-radical-generating impurities are present, suggesting that air exposure

is necessary but not sufficient for artifact production. Sample impurities combined with excessive air exposure may explain some of the high levels mentioned above, and therefore may be the basis of "phenol confusion."

In addition to phenol, we have found artifacts to be associated with lengthy digestion of protein during DNA extraction and lengthy hydrolysis of DNA to nucleotides. The latter artifact could be suppressed by the inclusion of desferal, which chelates traces of redox-active iron; hence we include the chelator in all of our solutions. At a concentration of 1 mM, desferal can interfere with oxo^{8}dG analysis. Yang and Schaich (32) report that desferal concentrations less than 0.06 mM fail to protect DNA from strand breaks, and that more than 0.1 mM desferal increases DNA damage. The free iron concentration in biological fluids is ≈0.005 mM (33). These findings combined with our results suggest that 0.1 mM desferal adequately protects samples without producing interfering peaks. The same concentration of desferal in homogenizing buffers also contributes to minimizing baseline values.

We previously reported that the analysis of small quantities of DNA (<20 μg) may greatly increase the ratio of oxo^{8}dG/dG and hypothesized that artifactual *in vitro* oxidation may exert a disproportionate effect on small quantities of DNA (29). If the limiting factor in such oxidation were, for example, metal ions contributed by buffers or hydrolytic enzymes, then the ratio of metal to DNA would be higher in samples with little DNA. Indeed, very high oxo^{8}dG/dG ratios have resulted when less than 1 μg DNA has been analyzed (34), and oxo^{8}dG/dG has been observed to increase more than 5-fold when the amount of DNA analyzed was decreased from 46 to 10 μg (19). Higuchi and Linn (35), however, obtained very low measurements of oxo^{8}dG from 10-μg samples of mitochondrial DNA with the use of solutions from which metals had been removed by dialysis (S. Linn, personal communication), suggesting that removal of trace metals may be critical. We find that to analyze very small quantities of DNA without observing elevated oxo^{8}dG, it is important to pay meticulous attention to HPLC hygiene. For instance, in one series of experiments, completely disassembling and cleaning our HPLC autoinjector immediately before use and including multiple blanks to monitor for sample-to-sample carryover averted contamination of sample chromatograms and eliminated the problems associated with small quantity analysis. Ideally, the hydrolysis of >100 μg of DNA per sample is preferable because it allows a greater margin of methodological error.

Alternatives to HPLC-EC have been described but do not, in our view, improve on those described here. A method based on the enzyme guanase has been devised (21) in which guanine is degraded under conditions that are said to leave the oxidized base unaltered, but we find the results hard to interpret, because in our laboratory we have found that oxo^{8}Gua is in fact a substrate for guanase (unpublished observations). A ^{32}P-postlabeling method (34) generates very high estimates (13.3 oxo^{8}dG/10^5 dG). Yin *et al.* (36) describe a mAb-based ELISA technique that, when tested against HPLC-EC, yielded oxo^{8}dG values that were correlated with but higher than HPLC-EC values.

Oxo^{8}Gua and oxo^{8}dG excreted in urine or tissue culture media are used to estimate DNA damage rates *in vivo* and *in vitro* as discussed below. With respect to analysis of oxo^{8}dG and oxo^{8}Gua in urine samples, we found that (*i*) oxo^{8}Gua in urine presents particular difficulties, and a modified method for analysis of this species is required, (*ii*) an interfering peak associated with mAb columns can be avoided with the use of a modified HPLC protocol, and (*iii*) the inhibition of xanthine oxidase by allopurinol does not affect the excretion of oxo^{8}Gua in humans. This latter issue is important, because some oxidized nucleobase species derive from xanthine oxidase and therefore are of little use as measures of oxidative injury (24).

Commentary: Interpreting oxo^{8}dG and oxo^{8}Gua Values. Oxo^{8}dG and oxo^{8}Gua have been used to estimate two different quantities: the number of oxidative adducts in DNA, which is sometimes referred to as the "steady-state" value, and the rate of DNA oxidation *in vivo*. The former is a direct function of the measurement of oxo^{8}dG in extracted DNA and is only ambiguous to the extent that the measurements may be inaccurate. Estimates of the rate of oxidation, however, are dependent on measurements of excreted adducts and therefore may be compromised by a number of assumptions about DNA repair, cell division, cell turnover, nucleotide metabolism, and the contribution of mitochondrial DNA.

Steady-State Measurements of oxo^{8}dG in DNA. The magnitude of oxidative DNA damage in young rat liver, based on the lowest measured value from this study (0.04 oxo^{8}dG/10^5 dG), is considerable. If one accepts that oxo^{8}dG represents roughly 5% of all oxidative adducts (37), then the total number of adducts is approximately 24,000 per young rat cell. The published estimates of oxo^{8}dG in Table 1 range as high as 3 oxo^{8}dG/10^5 dG for young rat liver, which translates into 6 million oxidative adducts per cell. Although the variability among the estimates may represent real differences between animals, it seems likely that previous studies have overestimated DNA oxidation. Although our estimate of oxidative adducts has decreased considerably, a burden of 24,000 oxidative adducts per cell is equivalent to or higher than estimates of endogenous nonoxidative adducts (38–40) as well as adducts derived from known environmental or dietary carcinogens (41–43), suggesting that oxidative adducts are highly relevant genotoxic lesions.

The number of molecules of oxo^{8}dG in DNA is a function of: (*i*) *de novo* oxidation of guanine bases in DNA, (*ii*) the removal of oxo^{8}dG from DNA by repair, (*iii*) the dilution of unrepaired adducts during DNA replication as cells divide, and (*iv*) the availability of free oxo^{8}dGTP for incorporation into DNA during replication. At present it is unclear how these different pathways contribute to a cell's measured load of oxo^{8}dG. A further potential complication, which has thus far received little attention, is the presence of dead or dying cells within a tissue. For instance, the oxidation of DNA in apoptotic cells may increase the overall tissue value while having little deleterious biological effect.

As methods have improved, the range of estimates of oxo^{8}dG has fallen considerably, presumably because of the suppression of artifacts. At the same time, however, the ratio between old and young animals has remained roughly constant. Table 1 includes data from experiments comparing young and old rats, which generally found a 2- to 3-fold increase in the ratio of oxo^{8}dG/dG. In our most recent experiments, by using the modified chaotropic NaI method, a highly significant 2.75-fold increase in oxo^{8}dG was again seen with age (0.04 oxo^{8}dG/10^5 dG vs. 0.11 oxo^{8}dG/10^5 dG). It is paradoxical that an age-related increase has been detected in a number of experiments despite differences in the overall

Table 1. Steady-state levels of rat liver oxo^{8}dG: Phenol- vs. NaI-based DNA extraction

Extraction method	Rat ages, mo	oxo^{8}dG/10^5 dG	Reference
Standard (phenol)	2–4	2.5	19
	2–4	0.54	*Results*
	4/24	1.5/3.6	48
	4/24	3/8	49
	4/24/30	0.8/1.0/1.8	31
	3 wk/5/30	1.1/1.8/1.2	50
Chaotropic NaI	2–4	0.28	19
	4/26	0.04/0.11	*Results*

level of oxo^{8}dG. A number of earlier studies reported absolute age-related increases on the order of 1 oxo^{8}dG/10^5 dG, more than 10-fold greater than the difference we see now (0.07 oxo^{8}dG/10^5 dG). This apparent consistency in the ratio of old to young values suggests possible alternative explanations. (*i*) DNA from old animals may be more susceptible to oxidation during sample work-up because of physical or chemical differences in old rat DNA (an increased abundance of free redox-active metal ions in aged tissues could be such a difference, for example; ref. 44). (*ii*) The modified chaotropic method has, in both young and old animals, selectively eliminated small but highly oxidized pools of DNA in which the young/old ratio is preserved. This might occur, for example, during storage after DNA isolation or during washing of the DNA before hydrolysis. Although these possibilities currently are speculative, future experiments should address them.

It is impossible to rule out some contribution by methodological artifacts to even the lowest values; however, it can be said that these values are low and reproducible. If one were to accept the lowest current estimates as valid, there nevertheless would remain questions about their significance. How are adducts distributed between cells? Do cells that have recently divided have fewer oxo^{8}dG adducts (because of the repair or dilution of preexisting lesions), or more adducts (because of the exposure of DNA to oxidants during synthesis)? Moreover, we know very little about the distribution of DNA adducts *within* cells. Coding and noncoding regions of the genome may not be equally susceptible to oxidation, because of differences in chromatin structure and DNA exposure during transcription. It is also likely that oxidative adducts are differentially repaired, because the selective repair of transcribed sequences has been well established (45). Therefore, it is possible that oxidative adducts may primarily persist in nontranscribed regions and be of little significance to cells.

DNA Damage Rates. How would cells cope with a large number of adducts? What is the rate of *de novo* formation of oxidative adducts? What percentage are repaired daily? In an attempt to answer these questions, the excretion of oxidative adducts in urine has been monitored by HPLC-EC after immunoaffinity purification, and estimates of adducts excreted per cell per day have been made (11, 25, 26). In Table 2, the details of such calculations are shown, along with the assumptions involved. Intriguingly, the estimated rate of excretion for rats (74,000 oxidative adducts per cell per day) is roughly 6.5 times higher than that for humans (11,500 oxidative adducts per cell per day), a difference that correlates with the species' relative oxygen consumption (45, 46). (In the past, these calculations have been discussed in terms of "oxidative hits per cell per day.") Do these numbers actually reflect a rate of oxidation in rats that is 6.5 times higher than in humans? Moreover, are measurements of excreted adducts compatible with steady-state values?

Table 2. Oxygen consumption and daily urinary excretion of oxidized DNA residues: Rats vs. humans

Daily excretion	Rat* 2×10^{11} cells	Human† 5×10^{13} cells
oxo^{8}Gua (normal diet)	15,164 pmol‡	118,000 pmol
oxo^{8}Gua (NA-free diet)§	1,192 pmol‡	ND
oxo^{8}dG (normal diet)	111 pmol‡	28,300 pmol
oxo^{8}dG (NA-free diet)§	124 pmol‡	ND
oxo^{8}Gua + oxo^{8}dG (NA-free diet)§	1,316 pmol‡	146,000 pmol
All oxidative adducts (whole organism)	26,320 pmol‡	922,000 pmol
All oxidative adducts (per cell)	73,817 molecules‡	11,500 molecules

The number of DNA-containing cells in a 70-kg human has been estimated at between 0.8×10^{13} and 7×10^{13}; for these calculations we have assumed that the value is 5×10^{13} (45). The number of cells in a rat value was derived from this estimate on the basis of relative weight. The damage rate for the rat was calculated from adduct excretion on a nucleic acid-free diet (46). The value for humans was obtained by calculating the sum of oxo^{8}Gua and oxo^{8}dG detected in the 24-hr urine samples of two individuals (24). The urine values were divided by the estimated number of cells in the human body and multiplied by 20, an estimate of the proportion of the total oxidative DNA lessions represented by oxo^{8}dG (37). By using values obtained from rat data, we then corrected the estimates for dietary effects. ND, not determined.

*O_2 consumed cell^{-1}·day^{-1}, 10^{12}. Ref. 51.

†O_2 consumed cell^{-1}·day^{-1}, 10^{11}. Ref. 46.

‡Ref. 12.

§Nucleic acid-free diet.

In Table 2, the calculation of cellular excretion is illustrated. A number of assumptions are necessary in such calculations, including estimates of dietary contribution, estimates of the number of cells in rats and humans, and so on, as described. We currently estimate that there are about 24,000 oxidative adducts per young rat cell, with approximately 70,000 excreted per cell per day. The excess of excreted species suggests either that there is a 3-fold turnover of adducts in rat cells each day (70,000/24,000), or that there are other sources of urinary adducts. In past studies, the steady-state oxo^{8}dG/dG ratio in humans has not been found to be markedly different from rodent cells, yet the estimated excretion value (about 10,000 adducts per cell per day) is considerably lower, as seen in Table 2. At face value, this implies that fewer than half of a human cell's adducts are turned over each day. Viewed in this way, the data suggest that despite a lower rate of oxidation in humans, an equivalent steady-state level of adducts is tolerated. In effect, there may be a threshold number of adducts *below* which rodent or human cells do not engage in repair activities. It must be stressed that this scenario, although plausible, remains to be tested.

The interpretation of adduct excretion in urine is complicated by the fact that other sources of oxo^{8}dG and oxo^{8}Gua may exist. (*i*) Mitochondrial DNA is a target of oxidative damage and may contribute to the excretion rate through mitochondrial turnover and repair (2); (*ii*) the apoptotic death of cells, characteristically associated with the degradation of DNA, may also contribute adducts; and (*iii*) the oxidation of cytosolic and circulating nucleobase pools, and DNA fragments, are also potential sources of excreted adducts. All of these confounders would inflate the estimate of excreted adducts and therefore lead to an overestimate of the number of "hits" repaired each day.

On the other hand, urinary excretion may underestimate endogenous repair. Oxo^{8}dG and oxo^{8}Gua are sensitive to oxidation and may be destroyed *in vivo* before excretion, or they may be enzymatically catabolized via salvage pathways that operate on intact nucleotides. Lastly, because the actual *in vivo* products of the repair of oxo^{8}Gua have not been definitively identified in mammals, it is possible that repair intermediates other than oxo^{8}Gua and oxo^{8}dG are released and presently escape detection.

DNA Oxidation in Tissue Culture: An Internal Control for oxo^{8}dG Techniques. In culture, primary human diploid fibroblasts excrete oxo^{8}Gua adducts into the medium, dividing roughly every 2 days. Hence, with fibroblasts, oxo^{8}dG/dG ratios and adduct excretion rates can be calculated from a uniform population of cells growing at a defined rate. The inputs and outputs of this *in vitro* model are considerably simpler than the dynamics of an organism, and so they should be useful in balancing the cellular budget of oxo^{8}Gua adducts, because the predicted excretion of oxo^{8}dG and oxo^{8}Gua can be calculated from the number of cells, rate of division, and steady-state level of oxo^{8}dG during the growth period. Reliable assays of both steady-state and excreted oxidative adducts from tissue culture cells therefore may serve as mutual internal

controls, because it is unlikely that the types of artifacts that affect steady-state oxo^{8}dG and those that affect excreted oxo^{8}Gua adducts should conspire to yield a balanced equation. To date, however, balancing measurements of steady-state oxo^{8}dG and excretion values from tissue culture cells remains difficult. For example, by using the phenol extraction technique, the steady-state ratio of oxo^{8}dG/dG in actively dividing IMR-90 cells was measured at about 2 oxo^{8}dG/10^5 dG (about 5 million oxidative adducts per cell), yet the excretion of oxo^{8}Gua into tissue culture medium by the same cells corresponded to about 150,000 oxidative adducts per cell per day (300,000 per round of cell division) (47). These two estimates do not equate. If all oxo^{8}Gua adducts were removed from DNA during replication and excreted into the medium, one would have expected to see approximately 2,500,000 oxidative adducts excreted per cell per day. That the measured excretion rate is far lower than this implies either (*i*) that the majority of oxo^{8}dG adducts were not repaired during replication and instead were passed on to daughter cells, (*ii*) that the steady-state value (5 million adducts per cell) is too high, or (*iii*) that the value of excreted adducts (150,000 per cell per day) is too low.

Conclusions. Methods for DNA isolation continue to improve, with modification of the chaotropic NaI technique producing the lowest oxo^{8}dG values reported to date by using HPLC-EC. Despite the decreased baseline values, age-associated increases in oxo^{8}dG levels persist with old/young ratios close to 3:1. Although the use of urinary oxo^{8}Gua adducts to monitor DNA oxidation *in vivo* remains attractive, the results are difficult to interpret. Comparison of carefully controlled groups can minimize some of the confounders (such as dietary contributions), but the effect of experimental treatments on, for example, the rate of cell turnover will remain unknown and uncontrolled. Therefore, given our ignorance about the dynamics of nucleotides and their oxidative adducts in whole-body metabolism, studies of urinary excretion must be interpreted with relatively greater caution than studies of oxo^{8}dG in DNA. Finally, although the simultaneous measurement of both steady-state and excreted adducts from tissue culture cells has potential as a control system for testing the plausibility of analytical measurements, the techniques require further development and study.

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