Reduction of Oxidation during the Preparation of DNA and Analysis of 8-Hydroxy-2‘-deoxyguanosine

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · SEPTEMBER 1998

Impact Factor: 3.53 · DOI: 10.1021/tx980041x · Source: PubMed

2 AUTHORS:

Tim Hofer
Norwegian Institute of Public Health
38 PUBLICATIONS **2,707** CITATIONS
SEE PROFILE

Lothar Moeller
Alcatel Lucent
138 PUBLICATIONS **2,290** CITATIONS
SEE PROFILE

Reduction of Oxidation during the Preparation of DNA and Analysis of 8-Hydroxy-2′-deoxyguanosine

Tim Hofer and Lennart Möller*

Department of Biosciences, Unit for Analytical Toxicology, Karolinska Institute, SE-141 57 Huddinge, Stockholm, Sweden

Received March 2, 1998

The promutagenic base 8-hydroxy-2′-deoxyguanosine (8-OH-dG) in DNA is known to be formed from oxygen radical attack on 2′-deoxyguanosine (dG) as a result of oxidative stress. Formation of 8-OH-dG from dG during workup is strongly dependent on temperature and transition metals and is mediated by oxygen radicals. The 8-OH-dG formation at temperatures between 0 and 140 °C for 1.5 h in an "ultrapure" solution followed a third-order equation. Fe^{2+} in the nM range mediated the formation of 8-OH-dG from dG without addition of H_2O_2. Fe^{3+}, Cu^{+}, and Cu^{2+} were shown to have weaker oxidative effects in comparison to Fe^{2+}. The pH (5.0−9.0) had a very limited effect on 8-OH-dG formation. Acid phosphatase, which contains iron at its active site, caused the formation of 8-OH-dG, whereas alkaline phosphatase did not. Phenol was not found to be oxidative. Fe^{2+}-catalyzed formation of 8-OH-dG was completely blocked by the nitroxide 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), whereas DMSO, mannitol, and DMPO had a significantly weaker protecting effect. Catalase cleaved the dG molecule and was not suitable for use. A simple, fast, and inexpensive method for 8-OH-dG workup and analysis was developed, and the background level seen in liver from 13-week-old male Sprague−Dawley rat was 0.23 ± 0.020 8-OH-dG/10^5 dG, which is up to 200 times lower than reported values from some other methods and up to 26 times lower when compared to other reports using HPLC−EC methods. In summary, the TEMPO method reduces oxidation of dG to 8-OH-dG during workup by (1) using chemicals low in transition metals, (2) using a cold workup procedure, (3) limiting the incubation time, and (4) using the nitroxide TEMPO in all steps.

Introduction

Oxygen radicals damage biomolecules such as DNA, lipids, and proteins. In DNA, oxygen radicals may induce single- and double-strand breaks and oxidation of bases that can lead to mutations (*1*). Reactive forms of oxygen are created in vivo by activation of phagocytic cells, ionizing irradiation, UV light, mitochondrial respiration, catalytic activity of transition metals such as copper and iron, and enzymatic metabolism (*2*). It has been estimated that 100 000 oxidative hits take place on DNA per cell and per day in the rat (*3*). Over 20 base modifications caused by oxygen radicals have been identified, of which 8-hydroxy-2′-deoxyguanosine (8-OH-dG)[1] is believed to be the most frequently formed base lesion (*4*).

8-OH-dG is commonly detected by HPLC with electrochemical detection (HPLC-EC, Figure 1). Other methods exist such as GC/MS (*5*), LC/MS (*6*), immunoslot blot assay (*7*), postlabeling (^{32}P-HPLC (*8*), ^{32}P-TLC (*9*)), and fluorescent probe-HPLC (*10*). Unfortunately, these methods introduce different degrees of artifact oxidation during workup. When the DNA is extracted from the cell, the in vivo protecting enzymes and antioxidants are no longer present to take care of oxygen radicals and repair the damage. Furthermore, a low concentration of transition metals is usually present. Due to oxidation during workup, small changes in 8-OH-dG caused by oxidative stress in vivo can vanish due to the added formation of 8-OH-dG from the workup procedure. It is of utmost importance to reduce the workup oxidation as much as possible to be able to properly identify the differences in 8-OH-dG levels in tissues and evaluate the true background level. The use of phenol during workup has been reported to increase the 8-OH-dG levels (*11*),

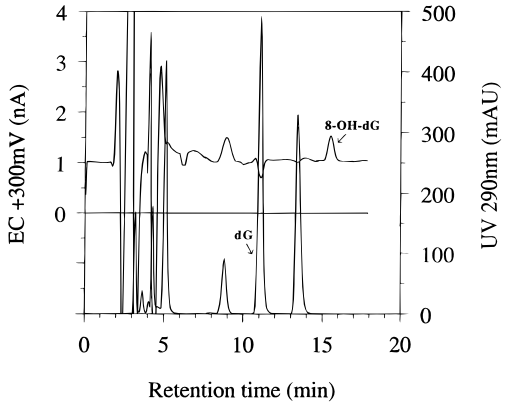

Figure 1. Representative HPLC chromatogram of hydrolyzed DNA analyzed by EC (upper) to detect 8-OH-dG and UV (lower) to detect dG. The 8-OH-dG peak represents 242 fmol. Details are given in the Experimental Section.

* To whom correspondence should be addressed. E-mail:lennart.moller@cnt.ki.se. Tel.: +46-8-608 91 20. Fax: +46-8-774 68 33.

[1] Abbreviations: dG, 2′-deoxyguanosine; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; EC, electrochemical detection; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

S0893-228x(98)00041-1 CCC: $15.00
Published on Web 07/23/1998

although these findings have been contradicted (*12*, *13*). We have recently reported that the use of [^{32}P]ATP in 8-OH-dG analysis mediated the formation of 8-OH-dG in the postlabeling procedure (*14*).

The aim of this study was therefore to identify factors that are responsible for the oxidation of dG to 8-OH-dG in the workup procedure and to develop a simple method that would inhibit oxidation.

Experimental Section

Caution: *TEMPO is highly toxic according to the manufacturer and is readily absorbed through the skin. Triton X-100 is a detergent for cell membranes. Phenol destroys proteins, and chloroform is a volatile solubilizer of hydrophobic substances. The use of gloves and a fumehood is recommended when handling these substances.*

Materials. The chemicals and materials used in the routine workup can be identified by the manufacturer's number that follows each name. It was verified that these chemicals were low in transition metals. Sodium acetate (4009-04) and methanol (8402) were from J. T. Baker (Phillipsburg, NJ). Zinc chloride (8816), magnesium chloride (5833), calcium chloride (2382), sodium chloride (6404), *o*-phosphoric acid (573), isoamyl alcohol (1.00979), sodium hydroxide (6498), ascorbic acid, iron sulfate heptahydrate, copper sulfate pentahydrate, and DMSO were from Merck (Darmstadt, Germany). Trichloromethane (32211) was purchased from Riedel-de Haën (Seelze, Germany). TRIS (0826) was from Amresco (Solon, OH). 8-OH-dG, dG, guanine, mannitol, ferric chloride hexahydrate, and Triton X-100 (X-100) were from Sigma (St. Louis, MO). Cuprous chloride was purchased from Fluka (Buchs, Switzerland), and redistilled phenol (IB05164) was from Eastman Kodak (New Haven, CT). Distilled water (549428) was from Pharmacia & Upjohn (Stockholm, Sweden), 2,2,6,6-tetramethylpiperidine-*N*-oxyl (42,636-9) and 5,5-dimethyl-1-pyrroline-*N*-oxide were from Aldrich (Milwaukee, WI), and vacuum hydrolysis tubes were purchased from Kontes (Vineland, NJ).

HPLC Analysis. The HPLC buffer, which consisted of 10% methanol, water of Milli-Q grade (Millipore, Bedford, MA), and 50 mM sodium acetate, was set to pH 5.3 with phosphoric acid and filtered through a CN 0.2 μm filter (Nalgene, Rochester, NY). The HPLC system consisted of a 0.2 μm mobile-phase filter (Scantec, Partille, Sweden), an isocratic HPLC pump (650, Scantec) set at 0.8 mL/min, an injector (7725i, Rheodyne, Cotati, CA) with a 200 μL loop, a guard column (10-02-00007, Optimize, Portland, OR), and two reversed-phase 3.9 × 150 mm, 5 μm, HPLC columns (Delta-Pak 011795, Waters, Milford, MA) connected in series. 8-OH-dG was detected with an electrochemical detector (Coulochem 5100A, ESA, Chelmsford, MA) with an analytical cell (5011, ESA; channel 1: 175 mV, channel 2: 300 mV) and a guard cell (5020, ESA; 350 mV). A UV detector (486, Waters) at 290 nm was used to detect dG, and data were recorded with MacLab (AD Instruments, Castle Hill, Australia).

dG and 8-OH-dG calibration curves were completed on each day of analysis, and three separate dG and 8-OH-dG weighings were performed before separate dilution. The purity and water content were corrected as follows: true quantity = weighed amount × purity × (1 − water content). Dilution was done in a buffer (50 mM sodium acetate in distilled water, pH 5.3) that resembled the HPLC buffer, and 250 μL aliquots were stored at −80 °C until analysis. Loop overloading (10%) was always used, with 220 μL injected into the 200 μL loop avoiding air bubbles, to ensure good reproducibility.

Incubations of dG. Incubations of 1 mL of 100 μM dG for 1.5 h under different conditions were made to test the formation of 8-OH-dG. The same buffer was used as had been used for nuclease P_1-enzymatic hydrolysis of DNA (50 mM sodium acetate, distilled water, 0.2 mM zinc chloride, pH 5.3). A temperature of 37 °C was used. Pink phenol was produced by placing the ultrapure phenol in the ambient air, unshielded from the light, and leaving it for 6 weeks at room temperature. When enzymes were tested, 250 μL of the solution was ultrafiltrated (UFC3LGC, Millipore, Bedford, MA) after incubation by centrifugation at 14 000 rpm for 10 min at 0 °C. Triplicate analysis was used throughout.

Preparation of Enzymes and Chemicals. Distilled water was used throughout. Ribonuclease A (R 6513, Sigma) was dissolved to a concentration of 100 μg/mL in 20 mM TRIS, 2 mM calcium chloride (pH 7.5). Protease (P 6911, Sigma) was dissolved to a concentration of 20 mg/mL in 20 mM TRIS, 2 mM calcium chloride (pH 7.5). Nuclease P_1 (27-0852-01, Pharmacia Biotech, Uppsala, Sweden) was dissolved to a concentration of 2.5 μg/μL in 50 mM sodium acetate, 0.2 mM zinc chloride (pH 5.3). These enzymes were stored in aliquots at −20 °C. The redistilled phenol was dissolved in 20 mM TRIS (pH 8.0) at 48 °C, and the pH was maintained at 8.0 with sodium hydroxide. The water phase was withdrawn, and fresh buffer was added. The phenol was stored in aliquots at −80 °C until used. Sevag consisted of isoamyl alcohol and chloroform (1:24). All solutions except sevag were stored in plastic bottles to avoid metal contamination from glass.

Optimized DNA Extraction Method. Male Sprague−Dawley rats (13 weeks old) were sacrificed using a guillotine. The livers were immediately removed, and the blood was quickly washed away with distilled water, after which they were frozen with CO_2 (s). The livers were stored at −80 °C until used. Microcentrifugation tubes (1.5 mL) (Treff, Degersheim, Switzerland) and pipet tips (Labsystems, Helsinki, Finland) were used in all steps thereafter. TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl, stored at 4 °C under argon) was dissolved in distilled water/methanol (5:1) and a 100 μM aliquot was freshly added to all solutions before use. All solutions were then chilled on ice, and each step, with the exception of enzymatic hydrolysis steps, was completed on ice. All centrifugations were made at 0 °C (5402, Eppendorf-Netheler-Hinz, Hamburg, Germany). Weighing of 80−100 mg of frozen Sprague−Dawley rat liver samples was accomplished in a cold room at 4 °C, and the tissue samples were immediately placed into a 1.5 mL tube and immersed in 950 μL of 0 °C homogenization buffer (1 mM magnesium chloride, 20 mM TRIS, pH 7.5), where they were manually homogenized with a pestle (749515-0000, Kontes, Vineland, NJ). After addition of 450 μL of homogenization buffer and centrifugation at 1400 rpm for 10 min, the supernatant was removed. The DNA-containing pellet was dissolved in 1.4 mL of the homogenization buffer and briefly vortexed to wash it, followed by centrifugation (1400 rpm, 10 min). The supernatant was withdrawn, and the pellet was dissolved for membrane separation on ice for 5 min in 1.4 mL of 0.5% (by volume) Triton X-100, 1 mM magnesium chloride, 20 mM TRIS (pH 7.5). After vortexing and centrifugation (1500 rpm, 10 min), the supernatant-containing membranes was discarded. This step was quickly repeated to wash the pellet. RNase-containing buffer (480 μL; 100 μg/mL) was added, and the tubes were incubated at 37 °C for 30 min and shaken every 10 min. Protease (12.5 μL) was then added totaling 500 μg/mL, and incubation was continued at 37 °C for 30 min and shaken every 10 min. The tubes were placed on ice, and 500 μL phenol was added. After being vortexed for 1 min and centrifugation at 14,000 rpm for 10 min, the water phase was transferred to a new tube. Further phenol was removed by sevag treatment twice. Sevag (500 μL) was added, the tubes were vortexed for 1 min and centrifuged at 14 000 rpm for 10 min, and the water phase was transferred to a new tube. Sodium chloride (60 μL; 5 M) and 1 mL of pure ethanol were added, and the tubes were then set at −20 °C for 10 min and centrifuged at 14 000 rpm for 10 min. After removal of the supernatant, the DNA pellet was washed with 1 mL of ethanol (70%), briefly vortexed, and centrifuged at 14 000 rpm for 3 min. The ethanol was removed with a pipet, keeping the tubes on ice after which the DNA hydrolysis buffer, 10 μL of nuclease P_1, and 2 units alkaline phosphatase (P 5521, Sigma; added directly from the manufacturers bottle and kept at 4 °C) were directly added to a final

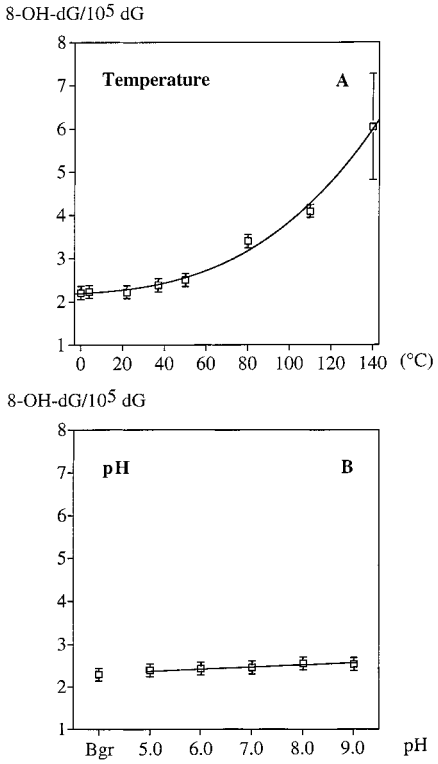

Figure 2. (A) Temperature dependence for 8-OH-dG formation from dG. Incubation (0−140 °C, 1.5 h) of 100 μM dG in "ultrapure" DNA hydrolysis buffer (50 mM sodium acetate, distilled water, 0.2 mM Zn^{2+}, pH 5.3). Each point represents three analyses and is shown as mean ± SD. The 8-OH-dG/dG ratio increase with temperature could be approximated with a third-order equation. (B) The effect of pH in the hydrolysis buffer (100 μM dG, 37 °C, 1.5 h). Each point represents three analyses and is shown as mean ± SD. The 8-OH-dG/dG ratio increased slightly with increasing pH. Background (Bgr) is the level of 8-OH-dG before incubation at pH 7.0.

volume of 240 μL. DNA hydrolysis was performed at 37 °C for 1.5 h, after which the solution was ultrafiltrated at 14 000 rpm for 10 min to remove enzymes. The tubes were frozen at −80 °C overnight. The solutions were only thawed immediately preceding analysis.

Results

The electrochemical detector can change slightly in day-to-day response, thereby requiring calibration standards of 8-OH-dG on the day of analysis. Although the UV detector displayed very little day-to-day variation, calibration standards of dG were also measured each day of analysis.

Incubations of dG. The temperature dependence for 8-OH-dG formation from dG was studied by incubation of dG in an "ultrapure" solution for 1.5 h, shown in Figure 2A. The 8-OH-dG/10^5 dG ratio increased in a third-order equation manner in relation to temperature. The 8-OH-dG/10^5 dG ratio was determined by comparison with calibration curves for dG and 8-OH-dG. At 80 °C and above, the dG molecule was hydrolyzed to guanine (detected by UV absorbance) and possibly to deoxyribose (not seen by UV), and here, the added amounts of dG (100 μM) were used in 8-OH-dG calculations. The hydrolysis of dG increased with temperature. Interestingly, 8-OH-dG was not hydrolyzed at 140 °C, showing a much stronger resistance to cleavage at the N-glycosidic bond than dG. The dG batch contained ~2 8-OH-dG/10^5 dG when delivered, i.e., the background level in the

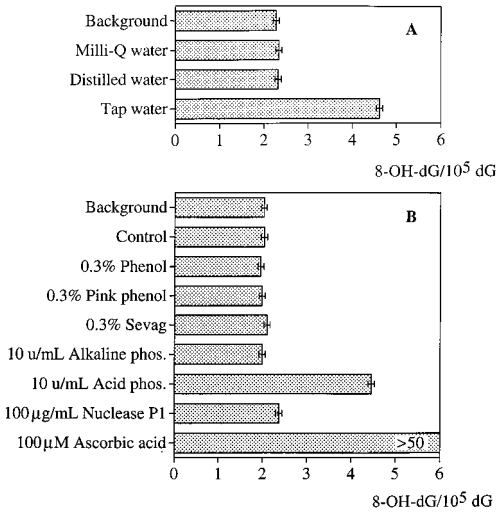

Figure 3. (A) Importance of water purity in the hydrolysis buffer (100 μM dG, 37 °C, 1.5 h). Each point represents three analyses and is shown as mean ± SD. (B) Test of phenol, pink phenol, sevag, enzymes used for DNA hydrolysis, and ascorbic acid. The concentration of phenol and sevag was 0.3 vol % in hydrolysis buffer (100 μM dG, 37 °C, 1.5 h). Each point represents three analyses and is shown as mean ± SD. Ascorbic acid produced over 50 8-OH-dG/10^5 dG, which was above the setting of the data sampling system. Background is before incubation and control is after.

diagrams. In the range of pH 5.0−9.0, 8-OH-dG formation was slightly lower at lower pH, although the pH-dependent difference in oxidation was very limited (Figure 2B). Although 8-OH-dG peaks could be seen at 5 fmol during calibration, a more realistic detection limit value is ~20 fmol due to base-line noise. The correlation coefficient was usually 0.98−1.00 for each calibration curve (minimum four points on each curve).

The effect of water quality is shown in Figure 3A. Tap water (used for comparison) catalyzed a formation of 2.3 8-OH-dG/10^5 dG in 1.5 h incubation at 37 °C, while no oxidation was induced by distilled or Milli-Q water vs background. Figure 3B shows that 0.3% phenol and 0.3% pink phenol did not catalyze 8-OH-dG formation when dG was incubated at 37 °C for 1.5 h. The pink color is likely a result of slow oxidation of phenol due to transition metals present, resulting in a pink substance (it took 3 weeks at room temperature for the phenol to show a slight pink color). Acid phosphatase catalyzed the formation of 8-OH-dG, whereas alkaline phosphatase did not. After the addition of 100 μM of ascorbate, production of 8-OH-dG could not be accurately quantified as the 8-OH-dG/10^5 dG ratio was above 50 and beyond the setting of the data sampling system (approximately 10 times the scale in the diagram, Figure 3B). Sevag had no effect on 8-OH-dG formation, and nuclease P_1 had a limited oxidative effect (Figure 3B).

Fe^{2+} (10 μM) dramatically oxidized dG to 8-OH-dG (37 °C, 1.5 h) without the addition of H_2O_2, whereas Fe^{3+}, Cu^+, or Cu^{2+} only showed slightly increased 8-OH-dG levels (Figure 4A). Zn^{2+} had no oxidative effect (data not shown). During the Cu^+ incubation, a dark substance quickly formed when CuCl was dissolved. Figure 4B shows the drastic dose-dependent increase in 8-OH-dG formation with Fe^{2+} concentrations higher than 10 nM, an oxidation that could be eliminated by keeping the tubes on ice or removing the oxygen by sparging with argon gas for 10 min using autosampler vials with septum.

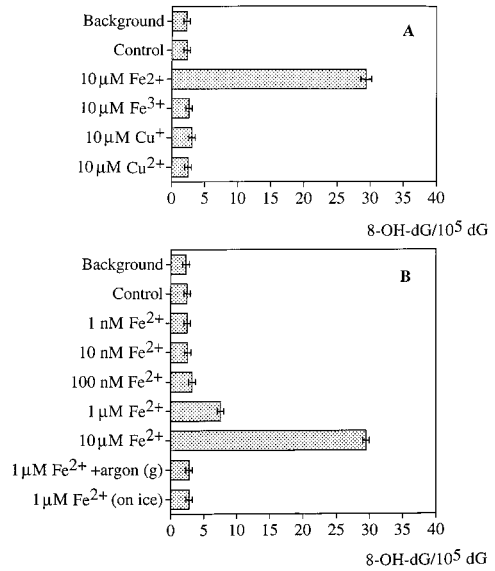

Figure 4. (A) Test effect of 10 μM of Fe^{2+}, Fe^{3+}, Cu^{+}, and Cu^{2+}, respectively, on 8-OH-dG formation in hydrolysis buffer (100 μM dG, 37 °C, 1.5 h). A dark substance formed in the Cu^{+} incubation (see text). Each point represents three analyses and is shown as mean ± SD. (B) Formation of 8-OH-dG from dG by catalysis of low concentrations of Fe^{2+} and inhibition of 1 μM Fe^{2+}-mediated formation of 8-OH-dG. Oxygen removal and incubation on ice drastically reduces oxidation of dG by Fe^{2+} in hydrolysis buffer (100 μM dG, 37 °C, 1.5 h). Each point represents three analyses and is shown as mean ± SD.

DMSO, mannitol, DMPO, and TEMPO were tested (37 °C, 1.5 h) to see if they would reduce 1 μM Fe^{2+}-mediated oxidation of dG to form 8-OH-dG (Figure 5A). TEMPO (100 μM) inhibited the 8-OH-dG formation completely. DMPO is hygroscopic, difficult to work with, and had less of a protective effect. DMSO and mannitol only had a slight protective effect on Fe^{2+}-mediated oxidation.

The heme (iron)-containing enzyme catalase can remove H_2O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$) or oxidize compounds with H_2O_2. Surprisingly, when incubated with dG only, the catalase cleaved the dG molecule forming guanine (G) and possibly deoxyribose (not seen by UV) and, hence, was not suitable for use under these conditions.

Shielding of normal light in the laboratory had no measurable effect on 8-OH-dG formation (data not shown).

Oxygen Removal during Analysis of DNA. An anaerobic box (constructed in the laboratory) with tight fittings for arms and continuously flushed with nitrogen gas was tested using sparging of liquids inside the box with argon gas. Every liquid had to be sparged with argon for at least 30 min to remove all oxygen before it could be used. This was problematic, especially with small volumes of enzymes. If a chilled centrifuge was placed inside the box, the box would get warm and conditions could vary from day-to-day.

Another approach used was to remove oxygen only before the warm enzymatic hydrolysis steps using 1.5 mL vacuum hydrolysis tubes. This approach required freezing the liquid before applying a vacuum due to "bumping" of the liquid and was, therefore, unsuitable when using enzymes. Sparging of the HPLC buffer with helium gas was not an available alternative as it strongly affected the electrochemical detection. However, sparging is probably not necessary as the time between injection and separation by the column is very short (seconds), and oxidation is not likely to be substantial in a buffer low

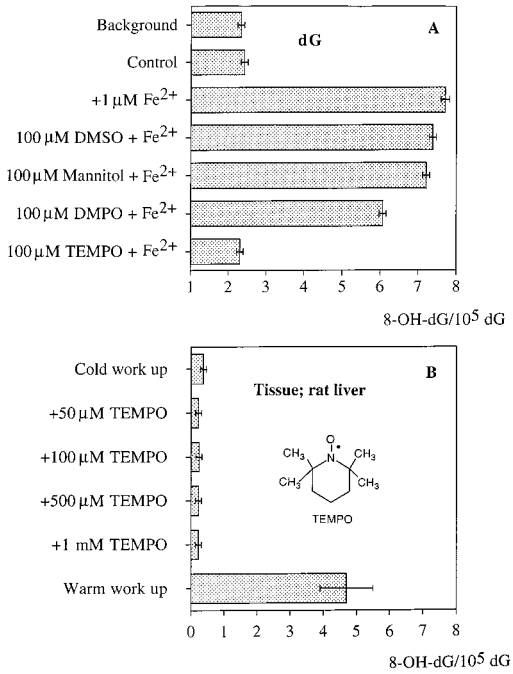

Figure 5. (A) Reduction of 1 μM Fe^{2+}-catalyzed 8-OH-dG formation using 100 μM of DMSO, mannitol, DMPO, and TEMPO, respectively (100 μM dG, 37 °C, 1.5 h). Each point represents three analyses and is shown as mean ± SD. (B) Analysis of Sprague−Dawley rat liver with different workup conditions. Cold (0 °C) workup conditions (0.37 ± 0.025 8-OH-dG/10^5 dG) ± additions of different concentrations of TEMPO to all solutions during workup (on average 0.23 ± 0.020 8-OH-dG/10^5 dG) compared to warm (23 °C) workup conditions (4.70 ± 0.80 8-OH-dG/10^5 dG). Each point represents three analyses and is shown as mean ± SD. In the warm workup procedure, an unchilled centrifuge was used, and the samples were handled at room temperature (23 °C). The chemical structure of TEMPO is shown in the lower (B) diagram.

Scheme 1. TEMPO Method (100 μM)

Homogenization	0 °C	Mg^{2+}
↓		
Nuclei preparation	0 °C	Mg^{2+} + Triton X-100
↓		
RNA cleavage	37 °C	RNase A, 0.5 h
↓		
Protein cleavage	37 °C	Protease, 0.5 h
↓		
Protein removal	0 °C	Phenol, sevag (twice)
↓		
DNA precipitation, wash	0 °C	Ethanol + NaCl
↓		
DNA hydrolysis	37 °C	Nuclease P_1 + alk. phos., 1.5 h
↓		
Ultrafiltration	0 °C	Protein removal
↓		
Freeze until analysis	-80 °C	Freezing
↓		
Thaw and inject	22 °C	Into HPLC-EC

in transition metals at room temperature. Overall, however, the different approaches for removing oxygen were very laborious and expensive for routine DNA extraction and were therefore not applied (data not shown).

Analysis of DNA. The TEMPO method was the procedure finally chosen to minimize oxidation during workup and analysis of 8-OH-dG in DNA (Scheme 1). The TEMPO method is defined by the use of the spin label TEMPO (100 μM) in all solutions, short incubation times, ultrapure solutions, and cold conditions during workup. This method was easy to use, produced pure DNA ($A_{260}/A_{280} = 1.82 \pm 0.02$), and yielded an average of 66.7 nmol dG from 100 mg of Sprague−Dawley liver. The magne-

Table 1. Reported Control Levels in Sprague−Dawley Rat Liver

method	8-OH-dG/10^5 dG ± SD	SD[a] (%)	ref
^{32}P-TLC	53 ± 20	36	9
GC/MS[b]	51 ± 10	20	26
HPLC/EC	6 ± 1	17	16
LC/MS	1−2		6
HPLC/EC	1.9 ± 0.4	22	27
HPLC/EC	1.5 ± 0.2	13	28
HPLC/EC	1.4 ± 0.4	28	29
HPLC/EC	1.1 ± 0.3	25	30
HPLC/EC	1.1 ± 0.6	52	31
HPLC/EC	0.6 ± 0.2	33	32
HPLC/EC	0.23 ± 0.02	8.6	*This study*

[a] Standard deviation in percent of the mean value. [b] In GC/MS 8-hydroxyguanine is measured. Lower 8-OH-dG levels have been reported for rat liver using GC/MS, but other papers having used Sprague−Dawley rat liver were not found.

sium ions bound to DNA and increased its weight, facilitating nuclei preparation at slow speed centrifugation. Twenty samples were prepared in 1 day, ready for analysis the following day. Contaminant hydrolysis of DNA with nuclease P_1 and dephosphorylation with alkaline phosphatase worked without complications. A representative chromatogram is shown in Figure 1.

An optimal reversed-phase HPLC chromatogram using electrochemical detection is depicted by the late appearance of the 8-OH-dG peak in the chromatogram where the base line is stable, is well separated from interfering peaks, is sharp to give a low limit of detection, and appears within a reasonable time span. If the base line is inclining or declining, the detector response will be too small. These requirements were fulfilled with the HPLC conditions stated here (Figure 1). Working on ice and using a chilled centrifuge (Figure 5B) markedly reduced the 8-OH-dG formation during workup (0.37 ± 0.025 8-OH-dG/10^5 dG) when compared to that seen under warm conditions (4.70 ± 0.80 8-OH-dG/10^5 dG). Addition of TEMPO to all solutions during workup decreased the 8-OH-dG formation further, with an average level of 0.23 ± 0.020 8-OH-dG/10^5 dG in normal Sprague−Dawley rat liver (Table 1). No significant differences in the 8-OH-dG/dG levels were observed for the different concentrations (50 μM−1 mM) of TEMPO.

Discussion

Because dG can be oxidized to 8-OH-dG, separate calibration curves must be used for calculations. Three weigh outs for dG and 8-OH-dG with separate dilutions decrease the chances of error due to calibration. The HPLC-EC system should also be optimized for maximum sensitivity by reducing the background noise as reported earlier (*15*).

Figure 2A shows that workup of DNA for 8-OH-dG analysis should be done as cold as possible. Enzymes, however, require higher temperatures. Especially the ultrafiltration, where dG is free in solution, is likely to be generating 8-OH-dG if an unchilled centrifuge is used. Boiling at 100 °C to separate the strands of DNA prior to enzymatic hydrolysis has been reported (*16*), a procedure that may induce oxidation. Incubations for 3 h (*6*) or overnight (*17*) have also been reported for enzymatic destruction of proteins in the workup procedure. Conditions such as these will likely lead to dG being oxidized to 8-OH-dG. As there is less 8-OH-dG formation in acidic conditions (Figure 2B), dG standards can be stored at pH 5.3 and as cold as possible. Methods dependent on acid hydrolysis of DNA and/or derivatization of DNA bases (GC/MS) could introduce errors in the analysis of dG and 8-OH-dG. First, the high temperatures used in the hydrolysis and derivatization step could promote 8-OH-dG formation. Second, hydrolysis of nucleosides to sugar and base constituents is dependent on energy, which was found to be higher for 8-OH-dG than for dG, which has also been reported (*18*). Hydrolysis efficiency of oxidized nucleotides and DNA adducts could differ from the undamaged nucleotide. LC/MS with enzymatic hydrolysis of DNA to nucleosides does not require breakage of the N-glycosidic bond.

It is possible that phenol of poor quality containing transition metals may have been used in reports stating that phenol was oxidizing during workup (*11*). Acid phosphatase contains iron at its active site (*19*, *20*), whereas alkaline phosphatase does not (*19*), which is probably the explanation for 8-OH-dG formation introduced by acid phosphatase (Figure 3B). Ascorbate has been reported to drastically enhance redox-cycling of the transition metals due to its reducing capacity (*21*). Several other antioxidants also have reducing properties and could therefore enhance 8-OH-dG formation.

Neither $^{\bullet}O_2^-$ nor H_2O_2 are believed to oxidize DNA constituents in the absence of transition metals (*5*). The same reactions observed with iron may occur for copper (*22*), chromium (*23*), vanadium (*24*), and other transition metals, but with different reactivities toward O_2 and H_2O_2. Zinc, however, is sufficiently stable as Zn^{2+}. As seen in Figure 4A, Cu^+ may not react with O_2 or, due to the dark substance that formed, may have formed metallic copper and copper(II) ions. It is also possible that Cu^+ formed an oxide or a complex. Fe^{3+} and Cu^{2+} will not react with O_2 or H_2O_2.

As mannitol only had a slight protective effect on Fe^{2+}-catalyzed 8-OH-dG formation when incubated with dG, both dG and mannitol having a concentration of 100 μM (Figure 5A), it can be speculated that the hydroxyl radical selectively attacks position C-8 on the dG molecule in preference to any site on mannitol. The most important protective effects of nitroxides such as TEMPO (structure in Figure 5B) are that transition metals are oxidized, keeping them in their unreactive form, and that they can react with $^{\bullet}O_2^-$ (*25*). Nitroxides do not react with either O_2 or H_2O_2 (*25*). TEMPO has been reported to have protective effects from a vast number of oxygen radical forming sources, both in vitro and in vivo (*25*). The addition of a substance to neutralize oxygen radicals and/or transition metals, thereby inhibiting oxidation of dG to 8-OH-dG during workup, is likely preferable to enzymatic removal of oxygen radicals. Whereas enzymes are pH and temperature dependent and must be removed before injection into the HPLC-EC, TEMPO can be added to all solutions, starting with the homogenization of the tissue sample and protecting dG all the way to the separation step on the HPLC column.

Metal chelators may protect metal-catalyzed oxidation of dG during workup, but difficulties may arise as metal chelators must bind the metal strongly, keeping it from redox cycling. Furthermore, as strong-binding metal chelators are specific to a certain metal, other metals present may only bind loosely and therefore accelerate redox-cycling and subsequent oxidation. EDTA and

deferoxamine, for instance (*21*, *22*, *24*), have been reported to enhance oxidation. Metal chelators may also form large complexes that can affect the analytical system. The addition of various electron-donating antioxidants to combat oxygen radicals in vitro would also be problematic due to their ability to reduce transition metals and possibly O_2 itself forming $^{\bullet}O_2^-$.

In Table 1, reported control levels of 8-OH-dG in Sprague−Dawley rat liver samples are shown. The more 8-OH-dG that forms during workup, the more difficult it is to identify differences between samples having low but varying 8-OH-dG levels. When the TEMPO-method was applied on Sprague−Dawley rat liver, a 200-fold decrease in 8-OH-dG levels was seen when compared to published ^{32}P-TLC and GC/MS methods. Compared to reported data on HPLC-EC, the TEMPO method produced 2.6−26 times lower 8-OH-dG/dG ratios (Table 1). When the standard deviation was calculated, expressed as a percent of the mean values, the TEMPO method's standard deviation was 8.6%, whereas other published data on the same type of control tissue was 13−52% (Table 1).

In conclusion, the TEMPO method is easy to use in the laboratory, is capable of a large number of workup/analyses per day, and does not require expensive DNA-extraction kits or equipment to work under an inert atmosphere.

Acknowledgment. This work was supported by the Swedish Medical Research Council (Contract No. 11567) and the Swedish Environmental Protection Agency. The authors wish to express their gratitude to Mary-Ann Zetterqvist for her skillful technical assistance, Dr. Dan Segerbäck for suggestions on DNA purification methods, and Dr. Magnus Zeisig for his help with chromatographic data acquisition.

References

(1) Breen, A. P., and Murphy, J. A. (1995) Reactions of oxyl radicals with DNA. *Free Rad. Biol. Med.* **18**, 1033−1077.
(2) Nivière, V., and Fontecave, M. (1995) Biological sources of reduced oxygen species. In *Analysis of free radicals in biological systems* (Favier, A. E., Cadet, J., Kalyanaraman, B., Fontecave, M., and Pierre, J.-L., Eds.) pp 11−19, Birkhäuser Verlag, Basel.
(3) Shigenaga, M. K., and Ames, B. N. (1991) Assays for 8-hydroxy-2′-deoxyguanosine: a biomarker of in vivo oxidative DNA damage. *Free Rad. Biol. Med.* **10**, 211−216.
(4) Wiseman, H., and Halliwell, B. (1996) Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem. J.* **313**, 17−29.
(5) Halliwell, B., and Dizdaroglu, M. (1992) The measurement of oxidative damage to DNA by HPLC and GC/MS techniques. *Free Rad. Res. Communs.* **16**, 75−87.
(6) Serrano, J., Palmeira, C. M., Wallace, K. B., and Kuehl, D. W. (1996) Determination of 8-hydroxydeoxyguanosine in biological tissue by liquid chromatography/electrospray ionization-mass spectrometry/mass spectrometry. *Rapid Commun. Mass Spectrom.* **10**, 1789−1791.
(7) Musarrat, J., Arezina-Wilson, J., and Wani, A. A. (1996) Prognostic and aetiological relevance of 8-hydroxyguanine in human breast carcinogenesis. *Eur. J. Cancer* **32A**, 1209−1214.
(8) Lutgerink, J. T., Graaf, E., Hoebee, B., Stavenuitez, H. F. C., Westra, J. G., and Kriek, E. (1992) Detection of 8-hydroxydeoxyguanine in small amounts of DNA by ^{32}P postlabeling. *Anal. Biochem.* **201**, 127−133.
(9) Devanaboyina, U., and Gupta, R. C. (1996) Sensitive detection of 8-hydroxy-2′-deoxyguanosine in DNA by ^{32}P-postlabeling assay and the basal levels in rat tissue. *Carcinogenesis* **17**, 917−924.
(10) Sharma, M., Box, H. C., and Paul, C. R. (1990) Detection and quantitation of 8-hydroxydeoxyguanosine 5′-monophosphate in X-irradiated calf-thymus by fluorescent postlabeling. *Biochem. Biophys. Res. Commun.* **167**, 419−424.
(11) Claycamp, H. G. (1992) Phenol sensitization of DNA to subsequent oxidative damage in 8-hydroxyguanine assays. *Carcinogenesis* **13**, 1289−1292.
(12) Kaneko, T., Tahara, S., and Matsuo, M. (1996) Nonlinear accumulation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative DNA damage, during aging. *Mutat. Res.* **316**, 277−285.
(13) Harris, G., Bashir, S., and Winyard, P. G. (1994) 7,8-Dihydro-8-oxo-2′-deoxyguanosine present in DNA is not simply an artifact of isolation. *Carcinogenesis* **15**, 411−413.
(14) Möller, L., and Hofer, T. (1997) [^{32}P]ATP mediates formation of 8-hydroxy-2′-deoxyguanosine from 2′-deoxyguanosine, a possible problem in the ^{32}P-postlabeling assay. *Carcinogenesis* **18**, 2415−2419.
(15) Adachi, S., Zeisig, M., and Möller, L. (1995) Improvements in the analytical method for 8-hydroxydeoxyguanosine in nuclear DNA. *Carcinogenesis* **16**, 253−258.
(16) Kim, K. B., and Lee, B. M. (1997) Oxidative stress to DNA, protein, and antioxidant enzymes (superoxide dismutase and catalase) in rats treated with benzo[*a*]pyrene. *Cancer Lett.* **113**, 205−212.
(17) Mitsui, T., Kawai, H., Nagasawa, M., Kunishige, M., Akaike, M., Kimura, Y., and Saito, S. (1996) Oxidative damage to skeletal muscle DNA from patients with mitochondrial encephalomyopathies. *J. Neurol. Sci.* **139**, 111−116.
(18) Chernikov, A. V., Usacheva, A. M., and Bruskov, V. I. (1996) Depurination of 8-oxo-7,8-dihydroguanine (8-hydroxyguanine) nucleosides. *Biochemistry (Moscow)* **61**, 35−38.
(19) Vincent, J. B., Crowder, M. W., and Averill, B. A. (1992) Hydrolysis of phosphate monoesters: a biological problem with multiple chemical solutions. *TIBS* **17**, 105−110.
(20) Sibille, J-.C., Doi, K., and Aisen, P. (1987) Hydroxyl radical formation and iron-binding proteins. Stimulation by the purple acid phosphatases. *J. Biol. Chem.* **262**, 59−62.
(21) Buettner, G. R., and Jurkiewicz, B. A. (1996) Catalytic metals, Ascorbate and free radicals: combinations to avoid. *Radiat. Res.* **145**, 532−541.
(22) Kennedy, L. J., Moore, K., Caulfield, J. L., Tannenbaum, S. R., and Dedon, P. C. (1997) Quantitation of 8-oxoguanine and strand breaks produced by four oxidizing agents. *Chem. Res. Toxicol.* **10**, 386-392.
(23) Faux, S. P., Gao, M., Chipman, J. K., and Levy, L. S. (1992) Production of 8-hydroxydeoxyguanosine in isolated DNA by chromium(VI) and chromium(V). *Carcinogenesis* **13**, 1667−1669.
(24) Shi, X., Jiang, H., Mao, Y., Ye, J., and Saffiotti, U. (1996) Vanadium(IV)-mediated free radical generation and related 2′-deoxyguanosine hydroxylation and DNA damage. *Toxicology* **106**, 27−38.
(25) Samuni, A., and Krishna, M. C. (1997) Antioxidant properties of nitroxides and nitroxide SOD mimics. In *Handbook of synthetic antioxidants* (Packer, L., and Cedenas, E., Eds.) pp 351−373, M. Dekker, New York.
(26) Wang, Y.-J., Ho, Y.-S., Lo, M.-J., and Lin, J. (1995) Oxidative modification of DNA bases in rat liver and lung during chemical carcinogenesis and aging. *Chem. Biol. Interact.* **94**, 135−145.
(27) Adachi, S., Kawamura, K., and Takemoto, K. (1994) Increased susceptibility to oxidative DNA damage in regenerating liver. *Carcinogenesis* **15**, 539−543.
(28) Adachi, S., Kawamura, K., and Takemoto, K. (1993) Oxidative damage of nuclear DNA in livers of rats exposed to psychological stress. *Cancer Res.* **53**, 4153−4155.
(29) Guo, N., Conaway, C. C., Hussain, N. S., and Fiala, E. S. (1990) Sex and organ differences in oxidative DNA and RNA damage due to treatment of Sprague−Dawley rats with acetoxime or 2-nitropropane. *Carcinogenesis* **11**, 1659−1662.
(30) Hirano, T., Yamaguchi, R., Asami, S., Iwamoto, N., and Kasai, H. (1996) 8-Hydroxyguanine levels in nuclear DNA and its repair activity in rat organs associated with age. *J. Gerontol.* **51A**, B303−B307.
(31) Fiala, E. S., Conaway, C. C., and Mathis, J. E. (1989) Oxidative DNA and RNA damage in the livers of Sprague−Dawley rats treated with the hepatocarcinogen 2-nitropropane. *Cancer Res.* **49**, 5518−5522.
(32) Umegaki, K., Ikegami, S., and Ichikawa, T. (1993) Influence of dietary vitamin E on the 8-hydroxydeoxyguanosine levels in rat liver DNA. *J. Nutr. Sci. Vitaminol.* **39**, 303−310.

TX980041X