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Optimization of the Workup Procedure for the Analysis of 8-Oxo-7,8-dihydro-2′-deoxyguanosine with Electrochemical Detection

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The artifactual generation of the biomarker for oxidative stress, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), during the workup procedure for its analysis is a difficult problem to solve, and the responsible factors are unclear. Here, peroxide removal and other antioxidant procedures during workup were compared using a limited amount of rat liver (50 mg) as starting material, with subsequent hydrolysis of 50 μg of DNA. A cold (0 °C) high salt GTC (4 M guanidine thiocyanate) nonphenol DNA extraction method was developed where DNA is quickly isolated. GSH (reduced glutathione) generated artifactual formation of 8-oxodG during the workup procedure, whereas H_2O_2 removal using catalase, Fe^{3+} removal and passivation using desferal, peroxide removal using glutathione peroxidase, ebselen and a peroxidase mimic lowered the 8-oxodG levels, all identifying peroxides as the responsible oxidants. Desferal was more protective when excluding Mg^{2+} and Ca^{2+} from buffers but was found to disturb the electrochemical detector when repeatedly injected five to six times, even at 100 μM. Addition of the OH$^\bullet$ scavenger ethanol in all steps at 2% v/v had no protective effect. Zn^{2+} was found necessary for efficient DNA hydrolysis using nuclease P_1, which was poor below 37 °C. Use of water substitutes was tested but inhibited DNA hydrolysis completely. $H_2{}^{18}O$ could, however, work for mass spectrometry methods. Long-term (38 days) storage of 0.5% v/v Triton X-100 generated more 8-oxodG than Tween 20 when incubated with free dG. The cold GTC DNA extraction method was used for analysis of freshly isolated human lymphocytes/monocytes from 60 healthy men using catalase and TEMPO as antioxidants, giving a background level of 0.074 ± 0.027 8-oxodG/10^5 dG (or 16 8-oxodG/10^8 nucleotides or 1943 8-oxodG/nuclei) which is probably the lowest value obtained yet. No increase with age was seen. Oxidation of dG to 8-oxodG during workup was found to fit a mathematically defined curve, and a calculated background level of 0.047 8-oxodG/10^5 dG was obtained. To obtain more reliable results it is recommended that control samples are included during the workup procedure, having an equal amount of cells (or DNA) as the exposed samples.

Introduction

Exposure of living cells to various oxidizing agents such as peroxides, singlet oxygen ($^1O_2$), UV- and γ-irradiation leads to an elevation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG)[1] in DNA resulting from oxidation of 2′-deoxyguanosine (dG). However, analyzing the true background 8-oxodG level in DNA and measuring small differences in 8-oxodG levels is difficult since dG can be oxidized during the workup procedure with artifactual formation of 8-oxodG (*1*, *2*). Coordinated comparisons of methodologies for analysis of 8-oxodG among mainly European laboratories are ongoing within ESCODD (*3*, *4*).

The workup procedure takes several hours and involves heat-dependent steps, such as the enzymatic hydrolysis of RNA, proteins, and DNA. The generation of artifactual 8-oxodG during workup is also influenced by the temperature, purity of solutions, incubation times, etc. (*5*). Small amounts of cells or DNA have been shown to give higher levels of 8-oxodG while keeping volumes and concentrations of enzymes constant (*6*−*8*), suggesting that for methodological comparisons the amount of cells and/or DNA used should be indicated.

The aim of this study was to develop a cold (0 °C) workup procedure, which to our knowledge has not yet been developed, and compare various antioxidant procedures for 8-oxodG analysis with electrochemical detection from a limited constant amount of cells (50 mg of rat liver) and DNA (50 μg) using the same set of calibration standards. It was considered important to evaluate the effect of peroxide removal, cold DNA extraction, and addition of a hydroxyl radical (OH$^\bullet$) scavenger molecule during the workup procedure for analysis of 8-oxodG. In addition, using the optimized analytical method, the background 8-oxodG levels in lymphocytes/monocytes from healthy men would be measured.

Experimental Procedures

Materials. The chemicals were stated low in transition metals by the suppliers. Sodium acetate, methanol, zinc chloride, magnesium chloride, calcium chloride, sodium chloride, glycine,

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[1] Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; dG, 2′-deoxyguanosine; dA, 2′-deoxyadenosine; rA, adenosine; EC, electrochemical detection; HPLC, high-performance liquid chromotography; GTC, guanidine thiocyanate; PLG, phase lock gel; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; UV, ultraviolet; GSH, glutathione (reduced form).

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glacial acetic acid, ascorbic acid, isoamyl alcohol, chloroform, dimethyl sulfoxide (DMSO), and 2-propanol were from Merck (Darmstadt, Germany). 8-OxodG, dG, dA, rA, calf thymus DNA, deferoxamine mesylate (desferal), 2-phenyl-1,2-benzisoselenazol-3[2H]-one (ebselen), glutathione (reduced form), Triton X-100 (X-100), RNase A (R-6513), protease (P-6911), catalase (*Aspergillus niger*, C-3515), and glutathione peroxidase (GPx, G-6137) were from Sigma (St. Louis, MO). Proteinase K and nuclease P_1 were from Roche Diagnostics (Mannheim, Germany). Glycerol and ammonium formate were from BDH (Poole, England). 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) and ethylene glycol were from Aldrich (Milwaukee, WI). Sucrose was purchased from J. T. Baker (Deventer, Holland), redistilled phenol from Eastman Kodak (New Haven, CT), Tris from Amresco (Solon, OH), guanidine thiocyanate (GTC) from Fluka (Buchs, Switzerland), Tween 20 (20605) from United States Biochemical (Cleveland, OH), Chelex 100 resin (100−200 mesh, sodium form) from Bio-Rad Laboratories (Hercules, CA), and Phase Lock Gel (PLG) tubes (prespun at 13000*g* for 2 min) from Eppendorf-Netheler-Hinz (Hamburg, Germany). 3-[4-(*N*,*N*-Dimethylamino)benzenetellurenyl]propanesulfonic acid sodium salt ("compound x") was kindly supplied by Lars Engman, Uppsala University, Sweden. Water was of Milli-Q grade. The software package MacCurveFit from Kevin Raner Software (Victoria, Australia) was used for mathematical curve fits.

Preparations. Aqueous solutions were chelex treated by stirring for 1 h and then filtered through a CN 0.45 μm filter (Nalgene, Rochester, NY) and stored in the dark in plastic bottles at +8 °C. Catalase and alkaline phosphatase were stored at +8 °C, and the other enzymes were frozen in small aliquots. Sevag consisted of isoamyl alcohol and chloroform (1:24). All steps and centrifugations were made at 0 °C if otherwise not stated. Ebselen was dissolved in 50% v/v ethanol (insoluble in pure water) and the other antioxidants in water prior to use. Solutions containing potential peroxide removal agents (catalase, GPx, ebselen, LE 1 and GSH) were preincubated at room temperature for at least 1 h in the dark immediately before chilling for 30 min on ice before use. Antioxidants were added to all solutions except 2-propanol and 70% v/v ethanol. To 4 M GTC (cold method) was added only TEMPO.

Test of Long-Term Storage of Detergents. Buffer containing 5 mM magnesium chloride and 20 mM Tris was set to pH 7.5 with hydrochloric acid and split into three parts. Triton X-100 and Tween 20 were added to 0.5% v/v, and the solutions were stored in the dark in plastic bottles at +8 °C for 38 days and were occasionally aerated. Incubations with 100 μM dG at 37 °C for 1.5 h tested for 8-oxodG formation.

Homogenization. Control male Sprague−Dawley rats (~275 g, corresponding to 49 days of age) were sacrificed, the livers (9−12 g) removed and rinsed with ice-cold PBS, and then cut into 1 g pieces which were immediately frozen under dry ice and stored at −80 °C. Twelve samples (two groups of six) were simultaneously extracted followed by DNA hydrolysis during each round of workup. Two pieces of liver (320−350 mg) were cut frozen on aluminum foil, put on ice, and separately homogenized in 10 mL of ice-cold homogenization buffer (20 mM Tris, pH 7.5, 5 mM magnesium chloride) using a 10 mL Potter-Elvehjem homogenizer with a PTFE pestle. A total of 50.0 mg was transferred to 6.0 mL tubes. Buffer was added to 4.5 mL, and the crude nuclei were pelleted at 1000*g* for 10 min at 0 °C. After withdrawing all the supernatant (contains membranes, proteins, mitochondria, and most of the RNA), the crude nuclei were dissolved in 1 mL of Tween 20 buffer (0.5% v/v Tween 20, 20 mM Tris, pH 7.5, 5 mM magnesium chloride) with a pipet, and buffer was added to 4.5 mL. After 5 min on ice, the crude nuclei/chromatin were pelleted at 1000*g* (must not be too slow) for 10 min at 0 °C. After withdrawal of the supernatant this procedure was repeated once with fresh 4.5 mL of Tween 20 buffer.

DNA Extraction/Warm Method. The crude nuclei pellets were dissolved in 540 μL of RNase A-buffer (100 μg/mL RNase A, 2 mM calcium chloride, 20 mM Tris, pH 7.5) using a pipet, and incubated at 37 °C for 30 min (shaken after 15 min). A total of 14 μL of proteinase K was added (20 mg/mL proteinase K, 2 mM calcium chloride, 20 mM Tris, pH 7.5) and incubated at 37 °C for 45 min (shaken after 15 and 30 min). The solution was transferred to a prespun 2.0 mL PLG (light) tube, and 560 μL of sevag was added. The tubes were handshaken using a "multishaker" for 1 min, and proteins/fat were removed at 13000*g* for 5 min. Another 560 μL of sevag was added, and the tubes were shaken and spun again (13000*g* for 5 min). After transferring the upper DNA-containing phase to a new 2.0 mL tube, 75 μL of sodium chloride (5 M) was added together with 635 μL of 2-propanol. After shaking, the DNA was precipitated at −20 °C for 15 min and spun down at 20800*g* for 10 min.

Scheme 1. Cold GTC Method

Homogenization, RNA removal	0°C	(Mg^{2+})
↓		
Nuclei preparation, RNA removal	0°C	(Mg^{2+}) +Detergent
↓		
Protein unfolding	0°C	GTC
↓		
Protein removal	0°C	SEVAG/PLG tubes
↓		
DNA precipitation, wash	-20°C, 0°C	Isopropanol, ethanol
↓		
DNA hydrolysis	50°C	Nuclease P_1 +alk. phos., 1 h
↓		
Enzyme removal	0°C	SEVAG/PLG tubes
↓		
Freeze until analysis	-80°C	Freezing
↓		
Thaw and inject	0°C	Into HPLC

DNA Extraction/Cold Methods. *4 M GTC.* The crude nuclei pellets were completely dissolved in 850 μL of cold (0 °C) 4 M GTC (20 mM Tris, pH 7.5) using a pipet, and the solution was transferred to a 2.0 mL PLG (heavy) tube (Scheme 1). The tubes were filled with sevag (≈850 μL), shaken by hand for 1 min and spun at 13000*g* to remove proteins/fat. The upper phase was transferred to a new 2.0 mL tube, and 850 μL of 2-propanol was added to precipitate DNA at −20 °C for 15 min. DNA was collected by spinning at 20800*g* for 10 min.

Density Gradient Nuclei Isolation Followed by 4 M GTC. OptiPrep (contains 60% w/v iodixanol) from Nycomed (Nycomed Pharma, Oslo, Norway) was used according to the manufacturer's instructions (application sheet S8), but at 0 °C. Frozen rat liver was cut into approximately 50 mg pieces and gently homogenized separately using a 7 mL Dounce glass-glass homogenizer (pestle A + B) in 4.5 mL of iso-osmotic buffer (0.25 M sucrose, 25 mM potassium chloride, 5 mM magnesium chloride, 20 mM tricine, pH 7.8). After filtration through a 40 μm Falcon cell strainer (Becton Dickinson, NJ), crude nuclei were spun down at 1000*g* for 10 min. The crude nuclei were dissolved in 500 μL iso-osmotic buffer and mixed with 500 μL 50% w/v iodixanol and transferred to a 2.0 mL tube. The solution (25% w/v iodixanol) was underlayered with 665 μL of 30% and 335 μL of 35% w/v iodixanol, respectively. Spinning (10000*g* for 20 min, swing-out rotor) caused the purified nuclei to collect at the 30%/35% iodixanol interface; they were then carefully withdrawn using a pipet. Preparation of the 50%/35%/30% iodixanol solutions was done with 150 mM potassium chloride, 30 mM magnesium chloride, and 120 mM tricine, pH 7.8. The purified nuclei were further processed using 4 M GTC as above.

TRI REAGENT. TRI REAGENT from Sigma which contains GTC/acid phenol was used according to the manufacturer's instructions but at 0 °C. A total of 320 mg of rat liver (cut frozen) was immediately homogenized for approximately 10 min in 3.2 mL of TRI REAGENT with a 10 mL Potter-Elvehjem homogenizer (PTFE pestle), and 550 μL (50.0 mg of liver) was transferred to 2.0 mL tubes. A total of 110 μL of chloroform was added, and the tubes were shaken and put on ice for 10 min. Spinning at 12000*g* for 15 min separated the liquid into three phases. After withdrawal of the top aqueous RNA-containing phase, 165 μL of ethanol was added to precipitate DNA (intermediate phase). The tubes were shaken and left on ice for 5 min, and DNA was spun down at 2000*g* for 5 min. All the supernatant (containing phenol and chloroform) was carefully removed, and the DNA was washed for 30 min in 0.1 M sodium citrate, 10% v/v ethanol. The DNA was spun down (2000*g* for 5 min) and the supernatant withdrawn.

DNA Wash and Hydrolysis. The DNA pellets were loosened from the tube wall and washed in 1.8 mL of 70% v/v ethanol by vortexing and spun down for 3 min at 20800*g*. After withdrawal of all the solution using a pipet, the DNA was dissolved by pipetting in 60 μL water (2 μL was taken to 118 μL of 10 mM Tris buffer, pH 7.4, for spectrophotometric concentration determination at 260 and 280 nm). A total of 50.0 μg of DNA was hydrolyzed in 100 μL of solution (final concentrations: 10 μg of nuclease P_1, 1 unit of alkaline phosphatase, 25 mM sodium acetate, 0.1 mM zinc chloride, pH 5.3) for 60 min at 50 °C in a 0.5 mL of prespun PLG (light) tube. A total of 100 μL of sevag was added, and the tubes were briefly shaken. Proteins were removed at 13000*g* for 5 min, and the upper phase was transferred to a new 0.5 mL tube which was stored at −80 °C. To test DNA hydrolysis at different temperatures, 50.0 μg of rat liver DNA (purified by the warm RNase A/proteinase K method) was hydrolyzed with 25 μg of nuclease P1 and 1 unit of alkaline phosphatase concurrently in 100 μL at the indicated temperatures.

Lymphocyte/Monocyte Analysis. Blood from 60 healthy men (average age: 52 years) was collected in two 8 mL sodium heparin Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ) which were spun at 1500*g* for 15 min at room temperature. The lymphocytes/monocytes were withdrawn, pooled into a 50 mL tube which was filled with ice-cold PBS, and spun down at 300*g* for 15 min at 0 °C. The cells were then washed twice by dissolving them in 50 mL of PBS with a pipet and spun down. After storage at −80 °C, approximately 20 samples were processed during each round of workup and analyzed the following day. Crude nuclei were prepared by pipetting in 1.8 mL of 0.5% v/v fresh Triton X-100 (20 mM Tris, pH 7.5, 5 mM magnesium chloride), leaving them for 5 min on ice and spinning the crude nuclei down at 1000*g* for 10 min. The supernatant was discarded and this procedure was repeated. DNA was extracted using 4 M GTC as described. After the PBS step, all the solutions contained 1 mM TEMPO and 50 units/mL catalase as antioxidants.

Lymphocyte/Monocyte Analysis (existing protease/TEMPO method). After PBS washing and crude nuclei preparation by pipetting in 0.5% v/v Triton X-100 buffer, the warm RNase A/protease method using phenol/sevag was used as previously described (2), however, with fewer cells and less extensive DNA solubilization. A total of 20 mL of blood was collected from 15 healthy humans in two 10 mL sodium heparin VENOJECT tubes (Terumo Europe, Leuven, Belgium). The blood was diluted in PBS, layered onto Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden), and spun at 400*g* for 30 min according to the manufacturer's instructions. After collecting the lymphocyte/monocyte layer, the cells were washed in PBS twice, split into three aliquots (each from 6.6 mL of blood) and processed as described (*2*). TEMPO (100 μM) was added to all solutions after the PBS wash steps.

HPLC/EC/UV Analysis. The HPLC system consisted of a zirconium mobile-phase filter (Elsico Labs, Moscow, Russia), an isocratic Scantec 650 pump (Scantec, Partille, Sweden) set at 0.8 mL/min with an extra PEEK pulse damper (Scientific Instruments, Inc., State College, PA), an injector (7725i, Rheodyne, Cotati, CA) with a 200 μL PEEK loop, a 1 mm (C-18) OptiGuard column (Optimize, Portland, OR), and two Delta-Pak (150 × 3.9 mm id, 5 μm) reversed-phase columns (Waters, Milford, MA). 8-OxodG was detected with an electrochemical detector (Coulochem II, ESA, Chelmsford, MA) with a graphite filter protected 5011 analytical cell (ESA, screen electrode: +200 mV, analytical electrode: +350 mV), and dG was measured with a 486 UV detector (Waters) set at 290 nm. Plastic and PEEK tubing was used throughout. The HPLC buffer consisted of 10% v/v methanol, water of Milli-Q grade (Millipore), 20 mM sodium acetate set to pH 5.3 with acetic acid and was filtered through a CN 0.2 μm filter from (Nalgene). A total of 100 μL (tubes were handthawed immediately prior to injection) was injected, and the retention times of dG and 8-oxodG (detection limit approximately 5 fmol for pure standard and 20 fmol for 50−100 μg of DNA) were 11 and 16 min, respectively. Calibration curves for dG and 8-oxodG were made by injection three times of each standard (100 μL, different concentrations). Preparation of dG and 8-oxodG standards was done by three separate weighings each before separate dilution. Correction was made for both the purity and water content using the following equation: true quantity = weighed amount × purity × (1 − water content). After use, the EC-detector was switched off and the buffer recirculated at 0.05 mL/min. Overnight HPLC-column washing (0.08 mL/min) using pure methanol (having the EC-detector disconnected), followed by 20 min HPLC-buffer reequilibration using fresh buffer at 0.5 mL/min before reconnecting the EC-detector, resulted in a lowering of the baseline current from 5−15 nA to 0.7−3.0 nA and fewer baseline fluctuations.

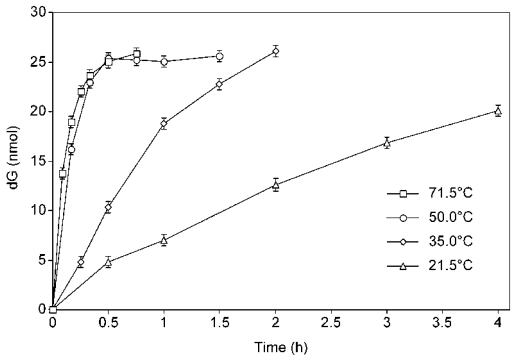

Figure 1. DNA hydrolysis curves. A total of 50 μg of rat liver DNA was hydrolyzed concurrently with 25 μg of nuclease P_1 and 1 unit of alkaline phosphatase in 100 μL of 25 mM sodium acetate, 0.1 mM zinc chloride, pH 5.3, at the indicated temperatures. Proteins were removed with sevag using PLG tubes. Hydrolysis overnight (21 h) at +4.0 °C and 0.0 °C gave 10.8 ± 0.1 and 7.3 ± 0.0 nmol, respectively. Duplicate analysis.

Check for RNA Contamination Using HPLC/UV. On a separate HPLC system, nucleosides from 5 μg of DNA were separated on a Genesis reversed-phase column (250 × 4.6 mm i.d., 4 μm) from Jones Chromatography (Hengoed, U.K.) using a linear gradient at 0.7 mL/min (Solvent Module 126, Beckman System Gold, Beckman Instruments, Fullerton, CA) changing from 98% v/v 50 mM ammonium formate, pH 4.6: 2% v/v methanol to 60% v/v 50 mM ammonium formate, pH 4.6: 40% v/v methanol over 30 min. rA and dA were measured at 254 nm (Diode Array Detector 168, Beckman System Gold) giving sharp separated peaks with retention times of 22.3 and 23.3 min, respectively.

Test of Water Substitutes. DNA pellet solubilization of 50 μg of calf thymus DNA in 100 μL of pure methanol, ethanol, ethyleneglycol, glycerol, and DMSO, all set to pH 4.5−6.0 with glacial acetic acid, was attempted by extensive pipetting. Also, DNA hydrolysis using nuclease P_1 and alkaline phosphatase was attempted after dissolving the DNA in aqueous hydrolysis buffer and adding each water substitute to 50% v/v, respectively. After attempted hydrolysis (50 °C, 1.5 h) the solutions were ultrafiltrated (UFC3IPH00, Millipore, Bedford, MA) at 12000*g* for 15 min to remove enzymes.

Results

DNA Hydrolysis. The tested temperature dependence for efficient DNA hydrolysis (Figure 1) using nuclease P_1 and alkaline phosphatase concurrently shows that DNA hydrolysis was very poor below 37 °C, consequently 50 °C for 60 min was selected for further experiments. Removal of Zn^{2+} from the hydrolysis buffer resulted in a 74% drop in hydrolysis efficiency, so it was retained. Lowering the nuclease P_1 amount from 25 to 10 μg had no major effect on DNA (50 μg) hydrolysis, and 10 μg was

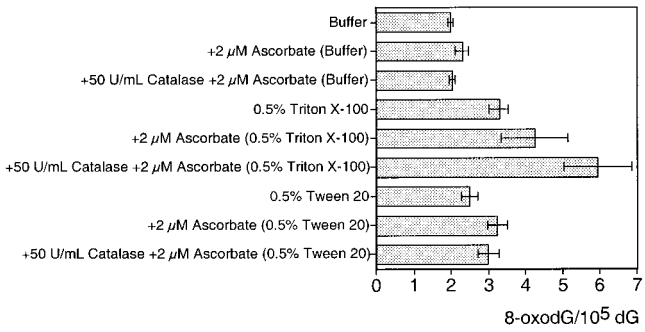

Figure 2. Test for 8-oxodG formation by incubation of 100 μM dG in long-term (38 days, +8 °C, dark, occasionally aerated) stored Triton X-100 and Tween 20 solutions at 0.5% v/v in 5 mM magnesium chloride and 20 mM Tris, pH 7.5, at 37 °C for 1.5 h. Triplicate incubation.

Table 1. RNA Contamination and DNA Yield for the Tested Methods

method	rA/dA (%)	μg of DNA/mg of liver[a]
RNase A/proteinase	0.56 ± 0.07	1.71 ± 0.01
GTC (0 °C workup)	17.7 ± 0.90	1.57 ± 0.11
Optiprep + GTC (0 °C)	10.5 ± 1.5	1.07 ± 0.12
TRI REAGENT (0 °C)	53.5 ± 1.3	1.63 ± 0.12

[a] RNA has been subtracted. Triplicate DNA extraction from 50 mg of rat liver.

also sufficient for >100 μg of DNA (Figure 5, panel B2). DNA pellet solubilization by extensive pipetting in pure methanol, ethanol, ethyleneglycol, glycerol, and DMSO was difficult, and DNA hydrolysis in all these water substitutes at 50% v/v resulted in no detectable dG peaks by HPLC/UV.

Detergent Tests. After the 38 days of storage, Triton X-100 was more oxidizing than Tween 20 when incubated with free dG (Figure 2). Catalase did not inhibit the 8-oxodG formation caused by the detergents. During workup, use of either 0.5% v/v Triton X-100 or 0.5% v/v Tween 20 gave equally pure DNA.

Rat Liver DNA Extraction Methods. The yield of DNA using Optiprep density gradient nuclei isolation followed by 4 M GTC was considerably lower than with the warm and the cold GTC methods (Table 1) and was not further used. Of the cold DNA-extraction methods tested, 4 M GTC worked best and also worked for rat brain and rat spinal cord (data not shown). RNA at these low concentrations was not found to disturb the detection of 8-oxodG or dG by HPLC/EC/UV (8-oxodG has a specific retention time and a specific oxidation potential). The DNA pellet from the warm RNase A/proteinase K workup procedure dissolved within a minute by pipetting in H_2O. For the cold GTC method, more extensive pipetting (5 min) was required, but the DNA was equally well hydrolyzed as detected by HPLC/UV. The PLG tubes made the upper phase withdrawal easy and very fast and increased the amount of liquid that could be withdrawn.

Effect of Antioxidant Procedures. The cold GTC method gave lower 8-oxodG levels (0.27 8-oxodG/10^5 dG) than the warm RNase A/proteinase K method (0.36 8-oxodG/10^5 dG) not using antioxidants (Figure 3). Inclusion of the tested antioxidants during the workup procedure had no significant effect on DNA yield, purity, or DNA hydrolysis efficiency. Use of 1 mM desferal during the initial DNA extraction steps significantly reduced the artifactual 8-oxodG formation when Ca^{2+} and Mg^{2+} were omitted (Figure 3). However, the use of 1 mM and 200

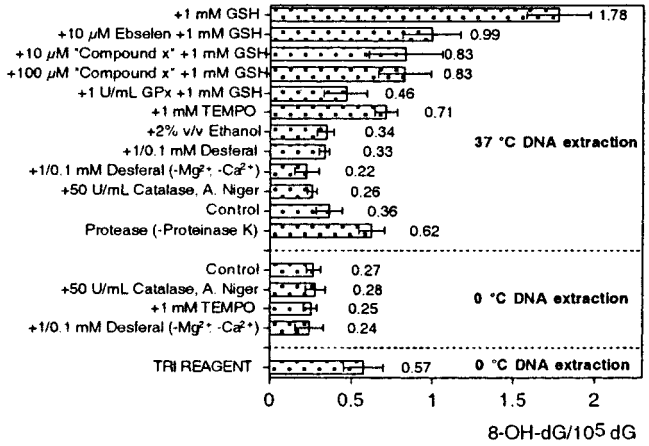

Figure 3. Analysis of Sprague−Dawley rat liver with different workup conditions. Each bar represents five to six separately processed samples and analyses from 50 mg of rat liver, each with hydrolysis of 50 μg of DNA. Data are shown as mean ± SD.

μM desferal during DNA hydrolysis resulted in immediate EC baseline disturbances. For 100 μM, severe disturbance was noticed after 5−6 injections with difficulties in quantifying the 8-oxodG peak. Preincubation of solutions with catalase at 50 units/mL lowered the 8-oxodG levels (0.26 ± 0.031 8-oxodG/10^5 dG) compared to no preincubation (0.33 ± 0.027) by direct comparison using the warm method. Catalase at 50 units/mL gave lower levels than 10 units/mL (0.32 ± 0.023 8-oxodG/10^5 dG) or 200 units/mL (0.34 ± 0.045 8-oxodG/10^5 dG). Inclusion of the $OH^{\bullet}$ scavenger ethanol at 2% v/v (0.34 M) in all the solutions had no significant effect on the 8-oxodG levels.

Analysis of Human Lymphocytes/Monocytes. Figure 5, panel A1, shows the 8-oxodG levels in lymphocytes/monocytes from 15 healthy humans using the existing protease/TEMPO method, with an average ratio of 1.03 ± 0.47 8-oxodG/10^5 dG (triplicate analysis each from 6.6 mL blood). Figure 5, panel A2, shows the data in Figure 5, panel A1, plotted against the amount of dG (nmole) detected. In all cases, high 8-oxodG/10^5 dG ratios were correlated with a low amount of dG (nmole). The concentration-dependence data fitted the curve $y = a + b/x$. If it is assumed that a relatively constant amount of 8-oxodG forms during the workup procedure then the following can be assigned: $y = (\text{8-oxodG}/10^5\ \text{dG})_{\text{measured}}$, $a = (\text{8-oxodG}/10^5\ \text{dG})_{\text{background}}$, $b = \text{8-oxodG}_{\text{workup}}$, and $x = \text{dG}_{\text{measured}}$. This gives the average amount of 8-oxodG generated by the workup to 20 fmol (= b) for the existing protease/TEMPO method.

The average background level in lymphocytes/monocytes from 60 healthy men was 0.074 ± 0.027 8-oxodG/10^5 dG (or 16 8-oxodG/10^8 nucleotides or 1943 8-oxodG/cell nucleus, calculating with 22% dG in DNA and 12 × 10^9 nucleotides/diploid genome) using the cold GTC method starting from 16 mL of blood with hydrolysis of all the extracted DNA, and TEMPO/catalase as antioxidants (the average 8-oxodG value for each individual was used from 2 to 4 separate workup procedures and analyses; total number of analyses: 214). No increase in 8-oxodG levels with age was observed (Figure 5, panel B1). Plotting the 8-oxodG/10^5 dG values from all the points of analysis (n = 214) against the dG amount measured by HPLC/UV (Figure 5, panel B2) and using the curve fit method ($y = a + b/x$) gave 0.047

8-oxodG/10^5 dG (= *a*) where the curve flattened out (or 10 8-oxodG/10^8 nucleotides or 1238 8-oxodG/cell nucleus) and 8-oxodG$_{workup}$ = 8.4 fmol (= *b*). This method gave 84.5 ± 32.8 μg of DNA (RNA subtracted), with an rA/dA ratio of 3.0 ± 0.82% from 16 mL of blood using Vacutainer CPT tubes from 10 individuals.

Discussion

DNA hydrolysis was very poor below 37 °C using nuclease P_1 (Figure 1). An extensive hydrolysis of DNA at low temperatures could be tested for using other enzymes, but is likely to require a long time and may not therefore lower the 8-oxodG levels.

Long-term storage of the commonly used Triton X-100 was more oxidizing than Tween 20 when incubated with free dG for 1.5 h at 37 °C, although relatively weakly 8-oxodG generating (Figure 2). Catalase did not inhibit the detergent generated 8-oxodG formation (Figure 2). Polyether detergents are peroxidized both in the pure state and in aqueous solution and contain hydroperoxides (*9*). Triton X-100 has been found to be considerably more oxidizing that Tween 20 (*10*).

The switching from protease to proteinase K during the warm method and chelex treatment of solutions resulted in low background 8-oxodG/10^5 dG levels (Figure 3), even when using a limited amount of tissue and DNA. With no antioxidant added, the cold GTC method reduced the 8-oxodG levels compared to the warm RNase A/proteinase K method. The cold GTC method has the potential to work below 0 °C with the addition of glycerol. The use of TRI REAGENT to extract DNA was the fastest method (no isolation of crude nuclei pellets) but resulted in considerable RNA contamination (Table 1) and elevated 8-oxodG levels (Figure 3). With this method it is difficult to remove all the phenol from the DNA, and it may be more suitable for RNA extraction. Although the initial crude nuclei preparation steps removed most of the RNA from the RNA-rich liver sample (Table 1), the cold GTC method (Scheme 1) could, although not necessary for HPLC/EC/UV measurement of 8-oxodG, be further optimized to remove more protein/RNA (facilitates DNA solubilization) by quick filtration of the DNA containing GTC solution using Micropure-EZ filters (Millipore), or using spin columns separating on size (such as Sigma S-3420 columns), selective DNA binding, use of spin columns separating on size (such as Sigma S-3420 columns) or selective DNA binding, acid phenol and selective RNA precipitation using lithium chloride or ammonium acetate after GTC removal. For GC/MS where DNA is hydrolyzed into free base and sugar constituents, a nearly complete removal of RNA is required as the same bases are present in RNA.

The lowering in 8-oxodG levels using desferal suggests that free catalytic iron is responsible for a major part of the oxidation of 2′-deoxyguanosine during the workup procedure. However, the necessity of Zn^{2+} for effective DNA hydrolysis and the EC baseline noise disturbances [similar to those observed by H. J. Helbock, et al. (*7*)] suggests that desferal should be excluded from the DNA hydrolysis step for good EC detection. The use of catalase, TEMPO, or combinations thereof, can be a good alternative to desferal during DNA hydrolysis to avoid this problem. The lowering in 8-oxodG levels using preincubation of solutions with catalase compared to no preincubation, gave evidence for the existence of H_2O_2 in the solutions before use. The increase in 8-oxodG when using GSH indicate the presence of peroxides being reduced in the buffers, but should not be seen as a proof of OH$^\bullet$, as two-electron reductions of peroxides may also occur (*11*). The alkyl aryl telluride "compound x" (Figure 4) has been shown to have thiol peroxidase activity (*12*). The use of GPx during routine workup is not realistic due to its very high cost even at 1 unit/mL. Figure 5, panels A1 and A2, shows that the inclusion of 100 μM TEMPO in the solutions did not eliminate the artifactual 8-oxodG formation. Also, the free radical TEMPO increased the 8-oxodG levels when incubated with proteinase K (Figure 3), but not when using the high salt method. TEMPO has previously been found to be protective against 8-oxodG formation (*2*, *11*). The measured background of ~0.25 8-oxodG/10^5 dG from 50 mg of rat liver with hydrolysis of 50 μg of DNA (Figure 3) is slightly lower than a recently reported background level of 0.34 ± 0.06 8-oxodG/10^5 dG from 100 mg of rat liver (*13*), using desferal as antioxidant and the commonly used chaotropic method (*14*, *15*).

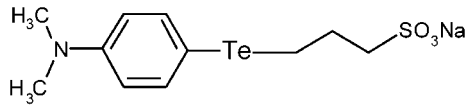

Figure 4. Structure of 3-[4-(*N*,*N*-dimethylamino)benzenetellurenyl]propanesulfonic acid sodium salt called "compound x" in the text to avoid acronyms.

Table 2. Measured Control Levels in Freshly Isolated Human Lymphocytes/Monocytes

method	8-oxodG/10^5 dG	±SD	ref
HPLC/EC	1	±0.35	*17*
HPLC/EC	0.52[a]	±0.10[a]	*18*
HPLC/EC	2.77	±0.18	*19*
HPLC/EC	6.8	±0.8	*20*
HPLC/EC	0.17	±0.1	*21*
HPLC/EC	0.074	±0.027	this study
oxygen removal during DNA extraction:			
HPLC/EC	1.157	±0.0414	*22*
HPLC/EC	0.188	±0.126	*23*
HPLC/EC	0.237	±0.121	*24*

[a] Calculated from Table 1 in this reference.

Switching to a solvent other than H_2O during the workup procedure could, based on knowledge on how 8-oxodG forms (*11*), be successful. However, as 50% v/v methanol, ethanol, DMSO, etc., were already found to inhibit DNA hydrolysis and as DNA was difficult to dissolve in these solvents, water substitutes are unlikely to work. Although expensive, isotopically labeled water $H_2{}^{18}O$ or $H_2{}^{17}O$ (both nonradioactive) could be an interesting choice for groups analyzing 8-oxodG (or 8-oxoG) by mass spectrometry methods such as LC/MS and GC/MS. After removal of dissolved O_2, any artifactual oxidation of dG (by formation of dG$^{\bullet+}$ with subsequent hydroxylation in $H_2{}^{18}O$, or $^{18}OH^\bullet$ attack (*11*) would be expected to give 8-(^{18}O)oxodG (instead of 8-(^{16}O)oxodG), which, having the mass M + 2, can be separated from 8-oxodG using mass spectrometry. The initial cytoplasmic H_2O could be removed by dilution or freeze-drying. Using D_2O instead of H_2O would not solve the problem as the protons in −OH, −SH, and, −NH groups are interchangeable with the solvent (*16*) [8-oxodG (keto form) is interconvertible with its tautomer 8-OH-dG (enol form)].

For the lymphocyte/monocyte samples, TEMPO/catalase was chosen as antioxidant due to possible EC-

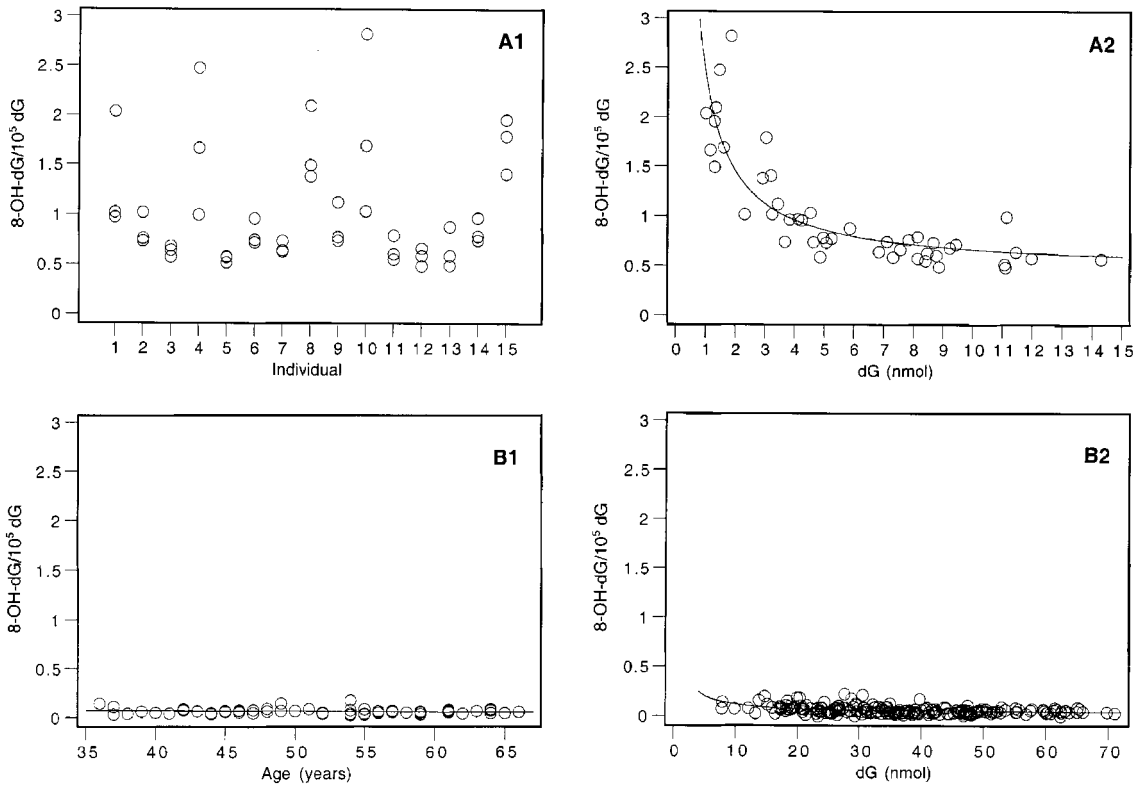

Figure 5. Freshly isolated human lymphocyte/monocyte background 8-oxodG levels by two different methods. (A1) DNA extraction from 6.6 mL of blood (triplicate) using the exising protease/TEMPO method (1.03 ± 0.47 8-oxodG/10^5 dG, $n = 15$ individuals). (A2) The data in panel A1 plotted against the measured amount of dG. The data could be fitted to the equation $y = a + b/x$. (B1) DNA extraction from 16 mL of male blood with hydrolysis of all the extracted DNA using the cold GTC method with TEMPO and catalase as antioxidats (average 0.074 ± 0.027 8-oxodG/10^5 dG). No increase with age was seen. Each point ($n = 6.0$) is the average of two to four analyses each from 16 mL of blood. (B2) Plot of the 8-oxodG values from all the analysis points ($n = 214$, each from 16 mL of blood) against the dG amount measured by HPLC/UV. Use of the curve fit ($y = a + b/x$) gave 0.047 8-oxodG/10^5 dG ($= a$) where the curve flattened out.

detector disturbances using desferal (*1*, *7*). The average background level of 0.074 ±0.027 8-oxodG/10^5 dG in human lymphocytes/monocytes is lower than previously reported levels (see Table 2). No indication of further oxidation of 8-oxodG into other species during the workup procedure was found. The absence of increased 8-oxodG/dG levels with age in lymphocytes/monocytes (Figure 5, panel B1) may be due to a large fraction of young cells.

The concentration dependence in Figure 5, panels A2 and B2, means, mathematically, that at higher dG concentrations the 8-oxodG formed during workup will be "diluted out" and the level of 8-oxodG/10^5 dG measured will approach the true background level. The difference in dG content is likely due to the large variance in lymphocyte/monocyte concentration among individuals. Comparison of curves for control and exposed samples could in many cases be better than comparison of 8-oxodG/10^5 dG ratios. In a perfect method, no 8-oxodG will be formed during the workup, and the curve $y = a + b/x$ should become a straight line: $y = a$ when $b = 0$, starting from a common source of material. Simultaneous workup and analysis of exposed and control tissue in equal amounts is recommended to reduce the influence of artifactual 8-oxodG formation (storage of cells/tissue at −80 °C has not been found to give 8-oxodG formation).

The recently developed "Fpg enzyme-HPLC/EC" assay (*25*) for 8-oxoG analysis gives cleaner chromatograms but requires the complete removal of RNA, a 100% dissolution of DNA to access all the 8-oxodG sites and may suffer from variability in enzyme activity.

In conclusion, the results give evidence that peroxides, with H_2O_2 specifically proven, are involved in the workup formation of 8-oxodG, and that the background 8-oxodG level in lymphocytes/monocytes from healthy men is lower than previously reported.

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