Method Development for Analysis of 8-oxodG as a Biomarker for Oxidative Stress

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Civ.ing. (kemi)

Stockholm 2001
Tillägnat den gods hälsan
Der Gesundheit gewidmet
Dedicated to good health
ABSTRACT

Oxidative damage to DNA gives oxidation of DNA bases and strand breaks that can cause mutations leading to cancer. Various forms of agents are capable of oxidising DNA in vivo including peroxides, singlet oxygen and UV- or \( \gamma \)-irradiation. Oxidation of DNA bases occurs mainly at dG, giving stable DNA adducts such as 8-oxodG and FapydG.

The use of 8-oxodG as a biomarker (expressed as the ratio 8-oxodG/dG) requires well-controlled workup conditions because 8-oxodG can be formed during the workup procedure. This may result in false high levels with difficulties in determining the true background levels, and differences between control and exposed tissue as a result. Existing methods require time consuming and warm (37°C) workup steps to extract and hydrolyse DNA before analysis. Since natural levels of 8-oxodG are low, a sensitive analytical tool is required when working with low mg amounts of cells.

In this thesis, methods for workup procedures and analysis of 8-oxodG in DNA using HPLC/EC/UV and \( ^{32} \)P-HPLC were developed. \( ^{32} \)P was found to strongly oxidise dG into 8-oxodG and a HPLC pre-separation step was developed before \( ^{32} \)P-postlabelling of 8-oxodG. Factors such as the purity of solutions, temperature, incubation time, peroxide removal and amount of cells during the sensitive workup procedure were found important, affecting the 8-oxodG/dG ratio. A fast (≈10 min) cold (0°C), high salt, non-phenol DNA extraction method procedure was developed to reduce the artifactual 8-oxodG formation. In addition, the use of catalase and the electron acceptor TEMPO was found to be protective against artifactual oxidation.

A new hydroxylation mechanism of carbon compounds by peroxides is suggested, where transition metals mediate the two-electron reduction of H\(_2\)O\(_2\), with one-electron oxidation of the reducing agent and compound respectively. This produces carbon radical cations, which are hydroxylated in water. For dG oxidation in a H\(_2\)O\(_2\)-ascorbate system, this mechanism is shown to be thermodynamically favourable to a one-electron reduction of H\(_2\)O\(_2\), producing OH\(^-\) and, in addition, added OH\(^-\) scavengers were found to be ineffective. Peroxide oxidation of DNA is likely, if forming DNA radical cations, to give mainly oxidation of G due to electron transfer in DNA, compared to systems generating the more randomly adding OH\(^-\) (such as \( \gamma \)-radiolysis of water).

Background 8-oxodG/dG ratios in human lymphocytes were found to be much lower than previously reported. Oxidation of dG to 8-oxodG during workup was found to be relatively constant and to fit a mathematically defined curve that can help in estimating the true background level and the artifactual formation of 8-oxodG.

As the degree of workup formation of 8-oxodG can vary on different days, it is important to include control samples during each round of workup and analysis.
LIST OF PUBLICATIONS

This thesis is based on the publications listed below, which will be referred to by their Roman numerals.


VI. Hofer, T. and Möller, L. Optimization of the workup procedure for the analysis of 8-oxo-7,8-dihydro-2'-deoxyguanosine with electrochemical detection. *Submitted.*
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>AP site</td>
<td>apurinic/apyrimidinic site</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>dG</td>
<td>2'-deoxyguanosine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC</td>
<td>electrochemical detection</td>
</tr>
<tr>
<td>ESR</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>FADH₂</td>
<td>flavin adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>FapydG</td>
<td>2-amino-6-(2'-deoxyribos-1'-yl)amino-4-hydroxy-5-formamidopyrimidine</td>
</tr>
<tr>
<td>Fe⁡⁺</td>
<td>ferrous iron</td>
</tr>
<tr>
<td>Fe⁢⁺</td>
<td>ferric iron</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione (reduced form)</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione (oxidised form)</td>
</tr>
<tr>
<td>HAsc⁻</td>
<td>ascorbate</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>¹⁰₂</td>
<td>singlet oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide</td>
</tr>
<tr>
<td>OH⁺</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>8-oxodG</td>
<td>8-oxo-7,8-dihydro-2'-deoxyguanosine</td>
</tr>
<tr>
<td>8-oxodGTP</td>
<td>8-oxo-2'-deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-tetramethylpiperidine-N-oxyl</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet (light)</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 BACKGROUND

Life is believed to have begun in an anaerobic environment and has gradually adapted to an aerobic milieu with the appearance of blue-green algae some two billion years ago. The adaptation to molecular oxygen (O$_2$) in the environment led to beneficial energy production but also to exposure to oxygen toxicity. In the absence of a catalyst O$_2$ is fortunately inert to reactions with organic biomolecules$^{[1]}$, and is believed to freely diffuse inside cells.$^{[2]}$ Still, our cells are continuously exposed to several forms of toxic insults related to O$_2$. Exposure to chemicals such as peroxides, ozone (O$_3$), metals as well as UV- and γ-irradiation and conditions involving production of reactive oxygen species (ROS, such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), singlet oxygen ($^1$O$_2$), hypochlorous acid (HOCI), peroxynitrous acid (ONOOH), alkyl hydroperoxides (ROOH), peroxyl radicals (ROO$^\cdot$) and alkoxyl radicals (RO'$^\cdot$)) lead to oxidative damage of biomolecules. Even at rest, our cellular mitochondria are estimated to leak some 1–2% O$_2^-$ ($\textit{in vitro}$ studies$^{[3,4]}$) which can convert into H$_2$O$_2$.$^{[3,4]}$ Several enzymes also produce O$_2^-$ and H$_2$O$_2$. Oxidative stress is defined as when the oxidising events overwhelm the protective systems of the cell. On the other hand, for instance, ROS are believed to be important for cell function and activates signal transduction pathways$^{[4,5]}$, although these events are incompletely understood.$^{[4]}$ Radicals (compounds with an unpaired electron) involved in oxidative stress are short-lived and difficult to measure. Electron spin resonance (ESR) and chemiluminescence can be used to detect free radicals, but it is very difficult to apply these methods for $\textit{in vivo}$ analysis.$^{[6]}$ Therefore, analysis is performed on sufficiently stable end-products from oxidation of damaged biomolecules, such as lipids, proteins and DNA, or measurement of the redox state$^{[5]}$ using the GSSG/2GSH ratio. To determine oxidative stress in diseases, several such parameters are usually analysed. The presence of antioxidants in food is believed to be important to prevent oxidative stress.

The cellular genome is thought to be continuously exposed to toxic agents, leading to DNA base adduct formation and strand breaks, which can cause mutations initiating cancer. The DNA adduct 8-oxo-7,8-dihydro-2’-deoxyguanosine (8-oxodG), discovered by H. Kasai and S. Nishimura in 1984$^{[7]}$, is formed by oxidation of 2’-deoxyguanosine (dG). It has been shown to be weakly mutagenic and can be used as a biomarker for oxidative stress. However, 8-oxodG can also be formed as DNA is sensitive to oxidation
during the workup procedure. Workup problems for analysis of 8-oxodG have led to the establishment of ESCODD (European Standards Committee on Oxidative DNA Damage)\cite{8,9}, which today constitutes about 30 research groups in Europe with the aim of comparing different methods, reducing artifactual oxidation during workup, and reducing the variation in analysis to a minimum.

There is a growing interest in the understanding of radical chemistry, oxidative stress and oxidative DNA damage.\cite{10-17}

Figure 1. Structures of the nucleosides dG and 8-oxodG, respectively. According to NMR-studies, 8-oxodG is predominant over the tautomeric form 8-OH-dG (8-hydroxy-2'-deoxyguanosine).\cite{18} In DNA, being an anionic polymer consisting of two annealed single strands of nucleotides (phosphate esters of nucleosides), the 5'-hydroxyl group of one nucleoside is linked to a 3'-hydroxyl group of another nucleoside by a phosphodiester bridge (PO$_4^-$).

1.2 DISEASES AND REAGENTS ASSOCIATED WITH OXIDATIVE STRESS
Some examples of diseases, exposure to chemicals or radiation where significantly elevated levels of 8-oxodG have been found are:
Table 1.

<table>
<thead>
<tr>
<th>Disease/agent</th>
<th>Source</th>
<th>Analytical tool</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ageing</td>
<td>Rat organs</td>
<td>HPLC/EC/UV</td>
<td>[19]</td>
</tr>
<tr>
<td>Alzheimer's disease</td>
<td>Human brain (postmortem)</td>
<td>HPLC/EC / Immunohistochemistry</td>
<td>[20]/[21]</td>
</tr>
<tr>
<td>Asbestos</td>
<td>Human leukemia cell line (in vitro)</td>
<td>HPLC/EC/UV</td>
<td>[22]</td>
</tr>
<tr>
<td>Benzene</td>
<td>Human lymphocytes</td>
<td>HPLC/EC/UV</td>
<td>[23]</td>
</tr>
<tr>
<td>Cadmium</td>
<td>Human lymphoblastoid cell line (in vitro)</td>
<td>HPLC/EC/UV</td>
<td>[24]</td>
</tr>
<tr>
<td>Copper</td>
<td>Rat liver/kidney</td>
<td>HPLC/EC/UV</td>
<td>[25]</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Human serum (8-oxoG)</td>
<td>Immunoaffinity column followed by HPLC/EC</td>
<td>[26]</td>
</tr>
<tr>
<td>Fanconi anaemia</td>
<td>Human leucocytes</td>
<td>HPLC/EC/UV</td>
<td>[27]</td>
</tr>
<tr>
<td>H. pylori gastritis</td>
<td>Human gastric mucosa</td>
<td>HPLC/EC/UV</td>
<td>[28]</td>
</tr>
<tr>
<td>H2O2</td>
<td>Human lymphoblast cells (in vitro)</td>
<td>GC/MS</td>
<td>[29]</td>
</tr>
<tr>
<td>Iron +LOOH</td>
<td>Human fibroblast cell line (in vitro)</td>
<td>HPLC/EC/UV</td>
<td>[30]</td>
</tr>
<tr>
<td>γ-Irradiation</td>
<td>Rat liver</td>
<td>GC/MS</td>
<td>[31]</td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>Rat brain</td>
<td>Immunohistochemistry</td>
<td>[32]</td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>Rat heart</td>
<td>Microdialysate analysed with HPLC/EC</td>
<td>[33]</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Human brain (postmortem)</td>
<td>HPLC/EC/UV</td>
<td>[34]</td>
</tr>
<tr>
<td>Parkinson's disease</td>
<td>Human brain (postmortem)</td>
<td>GC/MS / Immunohistochemistry</td>
<td>[35]/[36]</td>
</tr>
<tr>
<td>UV-B</td>
<td>Mice/Human epidermis</td>
<td>Immunohistochemistry</td>
<td>[37]/[38]</td>
</tr>
</tbody>
</table>

Other examples of diseases and agents are given in Paper III and in a review[39].
2 CHEMISTRY OF ROS

2.1 PRODUCTION OF ROS

The energy necessary for human life comes from the enzyme-catalysed controlled combustion of food by O\(_2\), producing ATP and reducing agents such as NADPH with carbon dioxide (CO\(_2\)) and water (H\(_2\)O) as end products.

In cellular mitochondrial respiration, O\(_2\) enters mitochondria after diffusion through the cytoplasm and is reduced to H\(_2\)O (O\(_2\) +4e\(^-\) +4H\(^+\) → 2H\(_2\)O) by NADH and FADH\(_2\) formed in glycolysis, fatty acid oxidation and the citric acid cycle, by a series of electron transfers in the inner mitochondrial membrane, producing ATP. However, *in vitro* studies show that approximately 1–2% of the O\(_2\) is only partly reduced during respiration and forms O\(_2\)\(^{•-}\), which is turned into H\(_2\)O\(_2\) by superoxide dismutase (SOD).\(^3\)\(^4\) O\(_2\)\(^{•-}\) and H\(_2\)O\(_2\) can cross cell and organelle membranes.

Phagocytes of the immune system engulf foreign particles such as bacteria and attack them by ROS and other reactive molecules:\(^4\)\(^0\)

\[
2O_2 + \text{NADPH} \xrightarrow{\text{NADPH oxidase}} 2O_2^{•-} + \text{NADP}^+ + H^+ \quad \text{"respiratory burst"} \quad (1)
\]

\[
2O_2^{•-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (2)
\]

\[
\text{Cl}^- + H_2O_2 + H^+ \xrightarrow{\text{myeloperoxidase}} HOCl + H_2O \quad (3)
\]

\[
H_2O_2 + HOCl \rightarrow O_2 + H_2O + HCl \quad (4)
\]

\[
O_2^{•-} + HOCl \rightarrow O_2 + OH^- + Cl^- \quad (5)
\]

ROS produced by metabolism, such as enzymatically generated O\(_2\)\(^{•-}\) and H\(_2\)O\(_2\) or from toxic metabolites, such as redox cycling quinones producing O\(_2\)\(^{•-}\), will indirectly lead to damage.

Reduced transition metals such as iron can reduce O\(_2\):

\[
Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{•-} \quad (6)
\]
Cellular reducing agents can reduce non-chelated Fe$^{3+}$ (or Cu$^{2+}$, etc.) back to Fe$^{2+}$ (or Cu$^+$). In humans, many transition metals are critical for enzymatic functions but can be toxic at high doses.

**Peroxynitrite.** O$_2^-$ may react with nitric oxide (NO', an endogenous signalling gas) forming the oxidant peroxynitrite/peroxynitrous acid (ONOO'/ONOOH).

\[
O_2^- + NO' \rightarrow ONOO^- + H^+ \rightarrow ONOOH
\]  

(7)

**UV light** exposure from natural sunlight can activate endogenous photosensitisers (flavins, porphyrins, etc.) producing $^1$O$_2$, or directly ionise (release an electron, oxidise) organic compounds.$^{[41]}$ In 1879 A. Downes and T.P. Blunt suggested that sunlight could dissociate H$_2$O$_2$ (homolysis), in one of the first papers mentioning OH'.$^{[42]}$

\[
H_2O_2 \rightarrow 2OH'
\]  

(8)

Alkyl hydroperoxides (ROOH) can also produce OH'. Homolysis of H$_2$O$_2$ and ROOH takes place at ≤ 340 nm light (optimum at 254 nm).$^{[15]}$ Suggestion of OH' formation from non-peroxides by light, H$_2$O and chlorine gas (Cl$_2$) was made by O. Stern and M. Volmer in 1920.$^{[43]}$ Heating also homolyses peroxides.

**Ionising radiation** can produce ROS or directly ionise molecules. Exposure of water to ionising radiation (radiolysis) results in several reactive species.$^{[15]}$ The amount of chemical species produced or consumed per unit of absorbed radiation energy is called the G-value, which depends on the type and energy of radiation. For radiolysis of water by $\gamma$-radiation ($^{60}$Co) or high-energy electrons (pulse radiolysis using an accelerator), the G-values are:

<table>
<thead>
<tr>
<th></th>
<th>OH'</th>
<th>$e_{aq}$</th>
<th>H'</th>
<th>H$_3$O'</th>
<th>H$_2$</th>
<th>H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-value (µmol/J)</td>
<td>0.28</td>
<td>0.28</td>
<td>0.062</td>
<td>0.28</td>
<td>0.047</td>
<td>0.073</td>
</tr>
</tbody>
</table>

**Ultrasound** (sonolysis) produce OH':

\[
H_2O \rightarrow H' + OH'
\]  

(9)
Smoke contains ROS. Inhaled cigarette or exhaust smoke contains a substantial amount of ROS as well as other toxic species.

2.2 MECHANISMS OF ACTION

Peroxides (H$_2$O$_2$ and ROOH) and O$_2^-$ are unreactive towards most molecules, but peroxides oxidise organic compounds in the presence of reduced transition metals. The mechanism of action is not clear and several suggestions have been made. In 1860, C.F. Schönbein reported that hydroperoxyl HO$_2^-$ (protonated superoxide, today known to rapidly dismutate into H$_2$O$_2$ and O$_2$) oxidised several substrates in the presence of iron.[44]

In 1876, H.J.H. Fenton observed that ferrous iron (Fe$^{2+}$), but not ferric iron (Fe$^{3+}$), together with hydrogen peroxide modified tartaric acid into a substance later identified as dioxymaleic acid.[45,46] Iron was termed "catalytic" as a very small quantity of iron was required to bring about the reaction.[46] Since then, many compounds have been found to be modified by a mixture of H$_2$O$_2$-Fe$^{2+}$ and other reduced transition metals such as Cu$^+$, Cr$^{2+}$, Co$^{2+}$, V$^{2+}$, Ti$^{3+}$ and Ni$^{2+}$ (not Zn$^{2+}$) have been found to catalyse similar reactions.[10,47] This is commonly referred to as Fenton chemistry.[47] Frequently, hydrogens are replaced by hydroxyl groups (oxidation). In 1901 it was suggested by W. Manchot and O. Wilhelms that higher oxidation states of iron (Fe(IV) or Fe(V)) were involved in the reduction of H$_2$O$_2$.[48,49] In 1931 F. Haber and R. Willstätter suggested that OH$^-$ could be produced by one-electron reduction of H$_2$O$_2$ by HO$_2^-$ (today known as a very slow reaction in the absence of catalytic redox cycling metals), and that OH$^-$ could abstract a hydrogen (H$^+$) from a carbon-hydrogen bond, and initiate radical chain reactions.[50] In 1932 F. Haber and J. Weiss suggested OH$^-$ production by one-electron reduction of H$_2$O$_2$ by Fe$^{2+}$:[51,52]

\[
Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH' \tag{10}
\]

Their paper is not concerned with oxidation of organic compounds, however. This reaction (reaction 10) is sometimes called the Fenton reaction, although it was never written by Fenton.[47] Also in 1932, W.C. Bray and M.H. Gorin suggested that the ferryl ion (FeO$^{2+}$, an oxidising Fe(IV) species) could be formed in the decomposition of H$_2$O$_2$ by Fe$^{2+}$[53], but their paper was not concerned with the oxidation of organic substances either. In 1946, J.H. Baxendale, M.G. Evans and G.S. Park suggested that OH$^-$ from reaction 10 adds to carbon double bonds and can thereby initiate a polymerisation reaction.[54] Also, HOCl can be one-electron reduced by Fe$^{2+}$ (or O$_2^-$) to produce OH$^-$. 
OH• acts by abstracting an electron (e−) or hydrogen (H•), or adds to carbon double bonds. However, the production of the commonly assumed OH• as the reactive intermediate has been refuted\[^{[55-58]}\] and this idea has frequently been questioned and discussed.\[^{[47,59-62]}\]

Other suggestions than free OH• exist, such as the ferryl ion (FeO\(^{2+}\)), can also be written as Fe(OH\(^{2+}\))\[^{[56,57]}\], "bound OH•"\[^{[63]}\] and "site-specific" produced OH•.\[^{[64]}\] Also, H\(_2\)O\(_2\)-Cu\(^{+}\) has been suggested to act via a higher valence Cu\(^{3+}\).\[^{[55]}\] Another possibility could be a transition-metal mediated two-electron reduction of H\(_2\)O\(_2\), with one-electron oxidations of Fe\(^{2+}\) and the carbon compound respectively, producing carbon radical cations that are hydroxylated in H\(_2\)O as suggested in Paper V.

\[
Fe(H_2O_2)^{2+} + HR \rightarrow Fe^{3+} + 2OH^- + HR^{+}
\]  

(11)

\[
HR^+ + OH^- / H_2O \rightarrow [HR - OH]^+ - e^- - H^+ \rightarrow ROH
\]  

(12)

Depending on circumstances, Fe\(^{2+}\) or other transition metals may not be reducing enough to reduce H\(_2\)O\(_2\) by one-electron transfer to produce OH•, or this reaction may be too slow. A two-electron reduction of H\(_2\)O\(_2\) is thermodynamically more favourable than a one-electron reduction (Paper V). Other peroxides, if not sterically hindered, can also participate in similar reactions. Alkyl hydroperoxides (ROOH) are more oxidising for both one- and two-electron reductions than H\(_2\)O\(_2\).\[^{[65]}\]

O\(_2^-\) has a low reactivity towards most biomolecules, but may liberate Fe\(^{2+}\) from protein-Fe\(^{3+}\) stores, which can cause enzyme inactivation.\[^{[47,66]}\]

The autoxidation, defined as spontaneous direct combination at ordinary temperatures of a substance with molecular oxygen, of organic compounds by oxygen from the air is a free radical chain reaction. For lipid peroxidation:

\[
Initiation \quad R' + LH \rightarrow RH + L'
\]  

(13) or

\[
Fe^{2+} + H_2O_2 + LH \rightarrow Fe^{3+} + OH^- + H_2O + L'
\]  

(14)

\[
Propagation \quad L' + O_2 \xrightarrow{v. fast} LOO'
\]  

(15)

\[
LOO' + LH \xrightarrow{slow} LOOH + L'
\]  

(16)

\[
Termination \quad 2LOO' \rightarrow non \text{- radical products}
\]  

(17) or
Oxygen reacts \( (k \sim 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}) \) with many carbon-centred free radicals.\(^{[12]}\) Peroxyl radicals can, in addition to \( \text{H}^+ \) abstraction, one-electron oxidise compounds. Intermediate alkoxy radicals can undergo carbon chain fragmentation to produce aldehydes:

\[
R_i(COOH)R_2 + Fe^{2+} \rightarrow R_i(CO')R_2 + Fe^{3+} + OH^-
\]

\[
R_i(CO')R_2 \rightarrow R_i' + H(CO)R_2
\]

During lipid peroxidation, products such as \( \text{F}_2 \)-isoprostanes (from arachidonic acid) or aldehydes such as malondialdehyde and 4-hydroxynonenal are formed. These can be directly measured, or measured after having reacted with, for instance, DNA, giving stable DNA-adducts.

**Ionising radiation** can directly ionise biomolecules, and the resulting radicals can give hydroxylations, fragmentations, or react with \( \text{O}_2 \). The reactive species \( \text{OH}^\cdot, \text{e}^-_{\text{aq}}, \text{H}^+ \) produced by radiolysis of water will cause damage to most molecules.\(^{[12]}\) In proteins, ionising radiation leads to the modification of virtually all amino acids whereas \( \text{H}_2\text{O}_2-\text{Fe}^{2+} \) produce metal-binding site-specific oxidation of amino acids.\(^{[58,67]}\)

**UV radiation** directly ionises molecules or produces \( ^1\text{O}_2 \), which can add to (oxidise) double bonds of certain biomolecules (only G in DNA).

**Peroxynitrite/peroxynitrous acid** (ONO\(\cdot\) / ONOO\(-\)) reacts rapidly with carbon dioxide \((\text{CO}_2)\)\(^{[68]}\), forming the relatively strongly oxidising carbonate radical \((\text{CO}_3^\cdot\))\(^-\), or can, in the absence of \( \text{CO}_2 \), homolyse into \( \text{OH}^\cdot \) and the weaker selective oxidant, nitrogen dioxide radical \((\text{NO}_2^\cdot)\)\(^{[69]}\):

\[
\text{ONOO}^\cdot + \text{CO}_2 \rightarrow \text{CO}_3^\cdot^- + \text{NO}_2^\cdot
\]

\[
\text{ONOOH} \rightarrow \text{NO}_2^\cdot + \text{OH}^\cdot
\]
2.3 PROTECTION AGAINST ROS

Several cellular defence mechanisms exist to battle oxidative stress. These include enzymes and antioxidants against ROS, repair of damaged biomolecules\[^{70}\], adaptation (changes in expression of protective genes), apoptosis (programmed cell death) and necrosis (cell death). The compartmentalised, compact nucleus contains histones and polyamines which protect DNA.\[^{71,72}\] Knock-outs of antioxidant and repair enzymes in animals have been shown to give severe effects.\[^{10}\] The intracellular environment is maintained in a reducing state by the pentose monophosphate shunt producing NADPH (from NADP\(^+\)), which can reduce GSSG through glutathione reductase forming GSH. In humans, the selenium-containing glutathione peroxidase (GPx) is the major peroxide removal enzyme:

\[
2\text{GSH} + \text{H}_2\text{O}_2 (\text{or ROOH}) \xrightarrow{\text{GPx}} \text{GSSG} + 2\text{H}_2\text{O} (\text{ROH} + \text{H}_2\text{O})
\]

(23)

Within membranes the phospholipid hydroperoxide glutathione peroxidase (PhGPx) removes peroxides.\[^{73}\]

SOD removes \(\text{O}_2^-\):\[^{74}\]

\[
2\text{O}_2^- + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2
\]

(24)

Catalase is located in the peroxisomes, where the \(\text{H}_2\text{O}_2\) production is large:

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} 2\text{H}_2\text{O} + \text{O}_2
\]

(25)

Antioxidants retard autoxidation and fall into two classes (a) preventive antioxidants such as peroxide decomposers, metal ion deactivators or ultraviolet light deactivators (which reduce the rate of chain initiation) and (b) chain-breaking antioxidants such as the reducing agents \(\alpha\)-tocopherol (vit E), ubiquinone (Q 10), \(\beta\)-carotene, urate, ascorbate (HAsc\(^-\), vitamin C), and GSH (which capture one of the chain carrying radicals to yield products which either do not continue the chain or continue it with a low efficiency).\[^{75}\]

\[
\text{LOO}^- + \alpha - \text{TOH} \rightarrow \text{LOOH} + \alpha - \text{TO}^-
\]

(26)

Flavonoids, derived from vegetables, can have an antioxidant function and also react with \(\text{O}_2^-\).\[^{76}\] HAsc\(^-\) and Q 10 are believed to one-electron reduce \(\alpha\)-TO\(^-\) back to \(\alpha\)-TOH,
although no enzyme has been found for this mechanism. HAsc\(^{-}\) undergoes two successive one-electron oxidations, forming the ascorbate radical (Asc\(^{\cdot}\)) and dehydroascorbic acid (DHA), respectively. HAsc\(^{-}\) is consumed but DHA can be reduced back to HAsc\(^{-}\) by several enzymes utilising glutathione or NADPH.\[^{77}\] \textit{In vitro}, ascorbate has been found to be stable in the absence of transition metals.\[^{78}\] However, in solutions containing peroxides (which most solutions do), very low concentrations of transition metals are needed to oxidise ascorbate as these metals redox cycle. Free amino acids and nucleotides etc. can also act as antioxidants, reacting with ROS. In general, one-electron oxidations of organic biomolecules can to a certain extent be "repaired" by electron or (H\(^{\cdot}\)) donating reducing agents such as \(\alpha\)-TOH, GSH and HAsc\(^{-}\). UV produced \(^{1}\)O\(_{2}\) can be quenched by carotenoids such as \(\beta\)-carotene and lycopene.
3 OXIDATION OF DNA

It has been estimated that in the rat 100,000 oxidative hits on DNA per cell take place every day, based on urinary excretion of 8-oxodG and its steady-state level in DNA.\[^{79}\]

Note that urinary 8-oxoG, cut out from DNA by the major base-excision repair, was not measured. Over twenty base modifications caused by ROS have been identified, and 8-oxodG and FapydG especially have been found to be elevated during oxidative stress. It is believed that the higher frequency of mutations in mitochondrial DNA (mtDNA) compared to nuclear DNA (nDNA) is due to mitochondrial O$_2^-$ production. Often, the reported 8-oxodG/dG ratios are higher in mtDNA than nDNA,\[^{20,80,81}\] but similar levels have also been reported\[^{82}\]. However, since cells contain very little mtDNA compared to nDNA, and since mtDNA require longer workup procedures, the artificial 8-oxodG workup formation is likely to be very significant for mtDNA analyses.

3.1 MECHANISMS FOR OXIDATION OF dG

Oxidation of dG is known to occur for ionising irradiation (such as γ-rays and UV light), transition-metal mediated peroxide reduction (H$_2$O$_2$, ROOH, and ROOR\[^{83}\]) as well as for reactions with $^1$O$_2$.\[^{84,85}\] One of the ways to produce 8-oxodG is to incubate dG together with H$_2$O$_2$ and a reducing agent, such as HAAsc$^-$, in the presence of catalytic transition metals. Whereas the free concentration of transition metals \textit{in vivo} is virtually zero, DNA acts as a chelator for metals. Peroxides formed on DNA are short-lived. One-electron oxidation of dG gives the guanine radical cation (dG$^{+}$) which can be hydroxylated in water by OH$^-$ or H$_2$O leading to 8-oxodG.\[^{86,87}\] The intermediate 8-OH-8-H-dG$^+$ radical, which has been declared reducing in nature\[^{88,89}\], has been found to react with O$_2$ ($k = 4 \times 10^9$ M$^{-1}$ s$^{-1}$) or undergo a ring-opening reaction ($k = 2 \times 10^5$ s$^{-1}$) in the absence of O$_2$;\[^{90}\] The guanine radical cation (dG$^{+}$) can also be deprotonated into the neutral guanine radical dG(-H)$^+$ (free dG$^{+}$ has p$K_a = 3.9$)\[^{91}\], which is not thought to be hydroxylated in water leading to 8-oxodG\[^{90}\], but instead reacts with oxygen, leading to imidazolone and oxazolone products.\[^{89,92}\] G(-H)$^+$ may also react with proteins, forming DNA-protein cross-links.\[^{93}\] In DNA, proton transfer from dG$^{+}$ to pairing dC may also occur\[^{91}\], but G$^{+}$ formed in DNA duplex should retain more cationic character than would free dG.\[^{16}\] Also 8-oxodG$^{+}$ can deprotonate into 8-oxodG(-H)$^+$ (free 8-oxodG$^{+}$ has p$K_a = 6.6$).\[^{94}\] Free dG has a p$K_a$ value of 9.4\[^{18,91}\], and free 8-oxodG has p$K_a = 8.6$.\[^{118}\]

Purinyl radicals (A(-H)$^+$ and G(-H)$^+$) do not react rapidly with oxygen, whereas OH$^-$-
purine addunts (A-OH$^\cdot$ and G-OH$^\cdot$) do.[95] OH$^\cdot$ is not thought to abstract a H$^-$ from C-8 of guanine, but to add to double bonds.

Figure 2. dG oxidation mechanisms in double strand DNA based on published observations.[89,90,96-99]
Oxidation of DNA

\( \text{H}_2\text{O}_2\text{-Fe}^{2+} \) can also abstract a H\(^+\) from deoxyribose in DNA, leading to strand breaks.\(^{[15,16]}\)

Copper-ions have been found to cause a larger increase in DNA-base damage than iron-ions in a \( \text{H}_2\text{O}_2\text{-ascorbate} \) system.\(^{[64]}\) ESR studies of specific radicals using spin traps suggest that OH\(^-\) can be created by transition-metal catalysed one-electron reduction of \( \text{H}_2\text{O}_2 \) under certain conditions and attack DNA. However, evidence against OH\(^-\) exists\(^{[100]}\) (Paper V). DNA intercalation of copper-1,10-phenanthroline (an effective DNA nuclease) produced OH\(^-\) from \( \text{H}_2\text{O}_2\text{-ascorbate} \), whereas extremely weak signals from OH\(^-\) were produced in the absence of 1,10-phenanthroline, having copper present.\(^{[101]}\) ESR, however, does not give information about other competing reactions that could be more predominant, and has been criticised as the spin adduct formed can be formed in other ways than from OH\(^-\).\(^{[47,61]}\)

Ionising radiation produces non-specific ionisations and ionises the DNA components approximately in direct proportion to the number of valence electrons available and, based on this, about 45% of the ionisations should occur at the bases (with no specificity among the bases), and 55% at the sugar-phosphate groups.\(^{[102]}\) In DNA, the purines and pyrimidines have a very high reactivity towards the solvated electron \( \text{e}^-_{\text{aq}} \), OH\(^-\) and H\(^+\) (the rates being \( 10^8-10^{10} \text{ M}^{-1} \text{ s}^{-1} \)), whereas the ribose-phosphate moiety is reactive mainly towards OH\(^-\).\(^{[12]}\) OH\(^-\) is an electrophilic, highly reactive radical which can add to the double bonds of DNA bases, or abstract a H\(^+\) from DNA bases or deoxyribose sugars in the backbone of DNA. This gives rise to oxidation of DNA bases (a large number of products), strand breaks, cross-links in DNA or to proteins, and the very dangerous double strand breaks.\(^{[12,15]}\) \( \gamma\)-Irradiation of monocytes were found to yield more FapydG than 8-oxodG.\(^{[103]}\)

UV light is divided into UV-A (400–320 nM), UV-B (320–280 nm) and UV-C (280–200 nm). In addition to direct ionisation of DNA causing oxidation and cyclobutane pyrimidine photodimers etc., natural sunlight activates endogenous photosensitisers, causing most of the oxidative DNA damage by producing \( ^1\text{O}_2 \).\(^{[41]}\) \( ^1\text{O}_2 \) is known to react specifically with guanine at neutral pH\(^{[97,104]}\), and was shown by NMR to react with a 8-methylguanosine derivative forming an endoperoxide.\(^{[105]}\) Guanine endoperoxides are unstable\(^{[105]}\) and can lead to 8-oxodG formation.\(^{[97]}\)
3.2 REDUCTION POTENTIALS AND RADICAL TRANSFER IN DNA

Radicals vary widely in their thermodynamic properties, ranging from potent oxidising to potent reducing agents. Reduction potentials of radicals can be used to predict if a reaction can occur. Standard reduction potentials ($E^0$) for ions and molecules are determined against the normal hydrogen electrode (NHE, assigned 0 V, pH 0) at 25°C having all reactants at 1 M. For biochemists, however, the physiological pH is of more interest and reduction potentials at pH 7 referenced to NHE can be denoted $E^0\text{'}$. $E^0\text{'}$ values are commonly determined using pulse radiolysis, and are dependent on pH and temperature etc. *In vivo*, $E^0\text{'}$ values span the range from -0.30 V (NAD$^+$/NADH) to 0.82 V (O$_2$/H$_2$O) under normal metabolic circumstances,[106] but higher toxic oxidising species such as peroxides and OH$^-$ can also be formed.

---

Figure 3. Effects of solar radiation on cellular DNA. J. Cadet, *et al.* (1997).[97] Reprinted with permission.
The following $E^0$ values for DNA bases have recently been determined by S. Steenken, et al. by pulse radiolysis:

<table>
<thead>
<tr>
<th>Couple (ox./red. species)</th>
<th>$E^0$ (V, pH 7)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>dT(-H)$^\cdot$, H$^+$/dT</td>
<td>1.7</td>
<td>[107]</td>
</tr>
<tr>
<td>dC(-H)$^\cdot$, H$^+$/dC</td>
<td>1.6</td>
<td>[107]</td>
</tr>
<tr>
<td>dA(-H)$^\cdot$, H$^+$/dA</td>
<td>1.42</td>
<td>[107]</td>
</tr>
<tr>
<td>dG(-H)$^\cdot$, H$^+$/dG</td>
<td>1.29</td>
<td>[107]</td>
</tr>
<tr>
<td>8-oxodG(-H)$^\cdot$, H$^+$/8-oxodG</td>
<td>0.74</td>
<td>[94]</td>
</tr>
</tbody>
</table>

2'-deoxyribose is much more resistant to oxidation than the DNA-bases.[108] See tables of reduction potentials for other species.[106,109,110] $E^0$ values for transition metals vary widely depending on the coordinating environment.[109]

The likelihood for a reaction can be estimated by calculating the change in Gibbs free energy $\Delta G^0$ for a reaction. For a spontaneous process, $\Delta G^0 < 0$, but the reaction may still require a catalyst to occur. The $\Delta G^0$ for a reaction can be calculated by combining $\Delta G^0$ for half-cell reactions (Paper V). $\Delta G^0$ for each half-cell reaction against NHE can be calculated using

$$\Delta G^\circ = -nF E^\circ$$

(27)

where $n$ is the number of electrons transferred and $F = 23.06$ kcal V$^{-1}$ mol$^{-1}$. For non-standard conditions such as redox equilibria or other temperatures, the Nernst equation should be used.[5]

The removal of an electron from DNA results in an "electron hole" (radical cation). As the base G is the most easily oxidised component of unmodified DNA, electron transfer via hole migration through DNA often leads to the guanine radical cation (dG$^\circ$). Electron
transfer in DNA has been estimated to be faster than the competing "H₂O-trapping" mechanism. Based on the reduction potentials above, it can be said that, for instance dA⁺ would be able to oxidise dG, which has also been found to occur rapidly for the dinucleoside phosphate dApdG. Also, sugar C4'-radical initiated chain breaks (after H⁺ abstraction) by heterolytic cleavage of the sugar-phosphate bond produce enolether radical cations which can oxidise G. Also, longer range cationic radical transfer can occur, by a "multistep hopping mechanism", depending on the DNA-base sequence. G sites especially have been found to be predominantly oxidised, and long-range electron transfer has been found to give 8-oxodG. The observed order of reactivity of G sequences is GGGG > GGG > GG > GA > GT = GC[16], meaning that G clusters are particularly prone to oxidations and mutations, and the reduction potential of dG in DNA can be expected to be lower than for free dG. The 5'-G residue of 5'-GG-3' steps in DNA[116], and the middle G and 5'-G of GGG triplets are the most easily oxidised[117], which has also been verified by calculations of ionisation potentials (IP).[117-119] Apart from the lowering of ionisation potential for G in purine "clusters" which are due to intrastrand π-π interactions[108], interstrand proton transfer[17,102] within the base pair (from G⁺ to C) also decreases the ionisation potential.[108] Neutral radicals, such as G(-H)⁺ are not expected to be highly mobile.[102] As seen in Table 3 (reduction potentials), 8-oxoG sites are even more easily oxidised[94,96], but they are much less common than G. The position of 8-oxodG in DNA also affects its ionisation potential, and 5'-(8-oxoG)G-3' have been found to be more easily oxidised than 5'-G(8-oxoG)-3'.[119] One-electron oxidation of 8-oxodG in oligonucleotides has been found to give guanidinohydantoin.[96] It is not clear how far electron transfer takes place in DNA in vivo. Several hot spot codons of p53 tumour suppressor and ras proto-oncogenes contain GG sequences. UV-A irradiation of cultured cells has been found to give telomere shortening, and noncoding regions such as telomere and introns, which are G-rich, have been suggested to protect against oxidative stress.[120] Cellular reducing agents may also repair electron holes in DNA before modifications, such as hydroxylations, have occurred. OH⁺ (from γ-irradiation) or ¹O₂ additions to G are not repaired by electron transfer, however. H⁺-abstraction by G(-H)⁺ from 2'-deoxyribose, which can lead to strand breakage, is not likely to occur.[108]
3.3 FORMATION OF DNA STRAND BREAKS

H⁺-abstraction by OH⁺ from the deoxyribose moiety in DNA (especially position C-4) can give strand breaks. Several possible mechanisms, both aerobic (reaction with O₂) and anaerobic, have been suggested. Radical centres at position C5 or C6 in pyrimidines can give direct strand scission. However, the majority of nucleobase lesions do not lead to direct strand scission. For example, 8-oxodG does not lead to strand breakage, but its oxidation product is a candidate for piperidine-induced strand breakage and alkali-lability. Also the enzymatic repair of oxidised DNA-bases, with half-lives of 8.5–62.2 min (55.2 min for 8-oxoG) leads to strand breaks, which are quickly repaired within a few minutes.

3.4 MUTAGENESIS OF 8-oxodG

8-oxodG has been found to mainly give G → T transversions, and to be weakly mutagenic. The base 8-oxoG can rotate into a syn conformation and pair with A instead of C during replication. After replication 8-oxoG is cut out and replaced by T, thus:

\[
8\text{-oxoG} - \text{C} \rightarrow 8\text{-oxoG} - \text{A} \rightarrow \text{T} - \text{A}
\]

8-oxodG may also cause mispairs of adjacent bases during replication. Also, 8-oxodGTP from the cell pool could be incorporated into DNA opposite A causing A → C transversions. The cutting out of 8-oxoG (only the base) by N-glycosylases results in apurinic sugars (AP sites), which are miscoding. 8-oxodG has also been shown to affect the methylation of nearby cytosines.

3.5 REPAIR OF 8-oxodG

Most commonly 8-oxodG is repaired by base excision repair (BER) by glycosylases, cutting out only single bases, leaving the deoxyribose unit and thereby creating apurinic/apyrimidinic (AP) sites. The human 8-oxoguanine DNA glycosylase hOgg1/hMMH (called the MutM/Fpg protein in the bacteria E. coli) is thought to be the major repair enzyme for 8-oxodG. In E. coli, the MutY protein (human homolog hMYH) cuts out A if paired with 8-oxoG after replication, allowing the insertion of C before removal of 8-oxodG. In vitro, hOgg2 has been found to cut out 8-oxoG from 8-oxoG/A and 8-oxoG/G mispairs, and hNTH1 to cut out 8-oxoG from 8-oxoG/G.
mispairs.[135] The AP sites may be incised by an AP endonuclease or an AP lyase, cleaving the DNA strand on which the base was cut out. Some glycosylases can in addition also have endonuclease activity. A deoxyribophosphodiesterase (dRpase) cuts out the sugar moiety, a nucleotide is then added in the gap by DNA polymerase and the repair is completed by DNA ligase.[131] Nucleotide Excision Repair (NER) cuts out longer oligomers of single stranded DNA. Human 8-oxo-7,8-dihydroguanosine triphosphatase h8-oxo-dGTPase/hMTH1 (MutT protein in *E. coli*) prevents incorporation of 8-oxodGTP by hydrolysing it to 8-oxodGMP.[136] 8-oxodGMP has been found not to be phosphorylated by human cell extracts or human guanylate kinase, instead 8-oxodGMP was found to be dephosphorylated to 8-oxodG by a nucleotidase.[137]
4 COMMON TOOLS FOR ANALYSIS OF 8-oxodG

Two approaches are mainly used to measure 8-oxodG: (a) steady-state levels in DNA (formation minus repair), described here, and (b) total levels in urine. Urinary methods measure 8-oxodG or 8-oxoG, however, these could partly come from food. Starting from limited amounts of tissue requires very sensitive analytical tools. The chromatographic methods quantitate 8-oxodG (or G) after hydrolysis of DNA using enzymes or acid. The tools described below should be seen as complementary to one another, each having their advantages and disadvantages.

4.1 ELECTROCHEMICAL DETECTION

On-line EC detection after HPLC separation of nucleosides is the most commonly used technique for 8-oxodG detection, having a detection limit in the 2–10 fmol range and high accuracy. EC detection of 8-oxodG uses amperometry (the eluent flows past the electrode) or coulometry (flow through a porous graphite electrode, highest sensitivity as nearly all of the electroactive species will be oxidised or reduced). The applied potential is characteristic of the compound of interest. The current that is measured in an electrochemical reaction is proportional to the concentration of the species that is being oxidised (or reduced). Here, coulometric detection with a high sensitivity analytical cell was used, in which a screen electrode (0 – +200 mV) to oxidise disturbing substances is placed before the analytical electrode (0 – +350 mV). Each electrode has a nonporous palladium counter electrode. Electrochemical oxidation of 8-oxoG has been found to give several products.\textsuperscript{[98,138]} Although dG can also be analysed with EC, a UV detector requires less maintenance and is commonly used.
Figure 4. The HPLC/EC/UV system used. In the middle is the EC instrument (above) and the isocratic pump (below). To the right is the EC analytical cell (above) and the UV detector (below).

A current/voltage (C/V) curve is made for 8-oxodG, which tells what potentials should be applied. This should be done after installing new EC cells. An extra conditioning cell similar to the screen electrode can be put before the analytical cell to provide additional selectivity (not used here). Also, a guard cell can be put before the injector to oxidise electroactive materials in the mobile phase (found to be unnecessary and not used). Each cell should have a graphite filter and extra cells will also increase the system pressure. Some other DNA adducts are also electrochemically active.[139]
Figure 5. C/V curve for 8-oxodG made by injection of a constant amount of 8-oxodG and stepwise lowering the potential of the analytical electrode while having the screen electrode turned off. The screen electrode can also be tested in the same way.

4.2 MASS SPECTROMETRY METHODS

After a column separation step, the molecules are converted into gaseous ions using an ion source and separated based on their mass-to-charge ratio using a mass analyser. Thus, identification of a substance is done by the retention time of elution and its significant mass spectrum, giving a very high specificity. Examples of mass analysers are: quadrupole mass filters, ion trap and magnetic sector. They differ in resolution, sensitivity and cost. Often tandem MS-MS methods are used which gives the highest sensitivity as the noise is reduced in the second analyser. Calibration is usually performed using isotopically labelled standards injected simultaneously as the sample, to correct for spray fluctuations etc.\cite{140,141} MS methods have the advantage of being able to detect several DNA-adducts simultaneously.\cite{142}

4.2.1 GC/MS

For GC/MS, the DNA is hydrolysed into sugar and base moieties using acid, which are then derivatised to make the DNA bases volatile. The bases are then separated on a column at high temperatures, connected to a mass spectrometer.\cite{29} In the most commonly used ionisation technique, Electron Impact, electron bombardment of the compound under near vacuum expels an electron (electrostatic repulsion), forming a positive molecular ion that fragments. Thus far, GC/MS has usually given the highest levels of 8-
oxodG among the methods. Most likely, the high temperatures (130–150°C) used for DNA hydrolysis are responsible for artifactual generation of 8-oxoG (see Papers II and V). DNA base derivatisation was previously also performed at high temperatures, but lately at room temperature.[141] GC/MS methods require a complete removal of RNA as G is present in RNA, and hydrolysis of the nucleotides as the N-glycosidic bonds differ in energy for different adducts vs. normal nucleosides, in order not to give false low or high levels (8-oxodG possesses a considerably more stable N-glycosidic bond that dG).[143] A detection limit of 18 fmol 8-oxodG for GC/MS has been reported.[144]

4.2.2 LC/MS
For LC/MS, electrospray is the most common ionisation technique.[145] An electrospray is produced by applying a strong electric field, under atmospheric pressure, to a liquid passing through a capillary tube with a weak flux. Highly charged droplets are formed and unfragmented or lightly fragmented ions are desorbed. LC/MS is a relatively new, promising technique for 8-oxodG detection that uses enzymes at lower temperatures to hydrolyse DNA and no derivatisation. Detection limits of 7.5[146] and 10[142] fmol 8-oxodG for LC/MS-MS have been reported.

4.3 THE COMET ASSAY
In the Comet assay (also called single cell gel electrophoresis)[123] single stranded DNA (seen as a "tail") migrates out from the nucleus ("head") in an agarose gel, which is quantitated after staining using a microscope. The background strand breaks are usually few and enzymes (glycosylases) are generally used to convert oxidatively modified DNA-bases to strand breaks. The commonly used enzyme, the Fpg protein from *E. coli*, also known as Fapy glycosylase, has at least three activities[147]: 1) a DNA glycosylase activity cutting out several substrates including 8-oxodG and FapydG[148], 2) an AP nicking activity that cleaves both the 3'- and 5'-phosphodiester bonds, and 3) deoxyribophosphodiesterase (dRpase) activity (excision of 5'-terminal deoxyribose phosphate). By adding other enzymes, other DNA adducts can be detected. An alkali step generates the necessary single-stranded DNA and also releases certain modified bases from the ribose unit. Strong alkali cleaves the DNA strands at the resulting AP sites as these have weakened sugar-phosphate bonds. Thus, the alkali step generates strand breaks in addition to the Fpg protein generated ones, but these can be subtracted by using no enzyme. The Comet assay is very sensitive as it can be used on single cells, but also has some drawbacks: it is less specific than most other methods as the Fpg protein excises
several DNA modifications (some also resulting from non-oxidative damage), it is not sure that all the modified sites have been excised, a group of 8-oxodGs in close proximity would be equivalent to a single DNA break as detected with the comet assay\cite{149}, variations in enzyme activity due to various reasons may give problems when comparing results over a longer time, and it works best for fresh, non-frozen cells or tissue. The comet assay usually gives lower levels of oxidative damage than the HPLC/EC/UV method.\cite{149} This could be due to these problems with the comet assay, or due to workup formation of 8-oxodG using the HPLC/EC/UV method.

4.4 IMMUNOHISTOCHEMISTRY

*In situ* detection of 8-oxodG using antibodies gives information on where in a tissue damage has occurred.\cite{37} After cell/tissue fixation, permeabilisation/slicing, RNase treatment and washing, an antibody recognising 8-oxodG in DNA is added. A secondary antibody conjugated with a fluorescent dye is added and detected using a microscope.\cite{150} A weakness with this method is that not all 8-oxodG sites may have been found and the antibody may also bind to base modifications similar to 8-oxodG.

4.5 POSTLABELLING METHODS

The basic $^{32}$P-HPLC method for lipophilic DNA-adducts capable of measuring $1$ adduct/$10^9$−$10^{10}$ normal nucleosides from ng-μg amounts of DNA was adapted for analysis of the polar 8-oxodG (Papers III and IV). Until now, 8-oxodG has been labelled with $[^{32}$P$]$ATP in the presence of dG with elevated 8-oxodG/dG ratios as result.\cite{151,152} For $^{32}$P-postlabelling methods, 8-oxodG should be labelled in the absence of dG as the radioactivity causes oxidation of dG into 8-oxodG (Paper I), requiring a pre-separation step. Although few groups use $^{32}$P-postlabelling for 8-oxodG quantification routinely, $^{32}$P-postlabelling methods have very high sensitivity and when hydrolysing small amounts of DNA these could be the only methods having a high enough sensitivity. $^{32}$P must be used under strict precautions due to the emitted radioactivity: protective equipment must be used and radioactive waste is also generated. The radioactive half-life ($T_{1/2}$) of the radionuclide $^{32}$P is 14.3 days due to $\beta$ emission, giving the stable daughter nuclide $^{32}$S.

$$^{32}P \rightarrow ^{32}S + e^- + \nu(\text{neutrino})$$

Fluorescent probe-HPLC also exists, but has poorer sensitivity.
4.5.1  $^{32}\text{P}}$-TLC

In thin-layer chromatography, $^{32}\text{P}$-labelled nucleotides are transported by a mobile phase through a stationary phase coated on a plate (called plate development). The mobile phase moves through the stationary phase by capillary action. In the commonly used three-directional TLC, a washing step is first employed after which the plate is cut and turned through 180°, after which the second step begins. After evaporating the solvent, the plate can be rotated 90° and developed with a second solvent. Quantitation can be done by measuring the radioactivity emitted from the 8-oxodG spot using a photographic film.$^{[152]}$ $^{32}\text{P}}$-TLC has a higher sensitivity than $^{32}\text{P}$-HPLC when analysing pure DNA adducts, but is more laborious and loses sensitivity when analysing complex mixtures like most in vivo samples. Removal of unmodified dGp by reacting with trifluoracetic acid to prevent artifactual formation of 8-oxodG during $^{32}\text{P}$-postlabelling has been tried, but gave only 77% removal of dGp.$^{[153]}$ Also, the reaction with DNA may itself be oxidising.

4.5.2  $^{32}\text{P}}$-HPLC

As described in detail in Papers III and IV, the necessary pre-separation of 3'-monophosphate deoxynucleotides (dNps) before $^{32}\text{P}$-postlabelling of 8-oxodG was developed using HPLC separation. This separation is also possible using capillary electrophoresis.$^{[154]}$ DNA was hydrolysed into dNps using micrococcal nuclease (MN) and spleen phosphodiesterase (SPD). Using an extra HPLC-column, low salt, no organic solvent (except for column washing) and pH 2.4–2.6 gave a good separation of 8-oxodGp from the other dNps, simplifying fraction collection of 8-oxodGp. After subsequent $^{32}\text{P}$-postlabelling of the 8-oxodGp fraction using T4 polynucleotide kinase (PNK) (requiring much less $[^{32}\text{P}}$ATP than when together with other nucleotides), nuclease P1 treatment to remove the 3'-monophosphate to decrease the polarity, [5'-$^{32}\text{P}$]8-oxo-pdG was analysed by HPLC at pH 3.4–3.6. A scintillation fluid is mixed into the HPLC buffer before the on-line detector, which converts the radioactive decay emitted from the $^{32}\text{P}$-labelled nucleotides into light which is detected. The measured signal is converted into the amount of 8-oxodG. In Paper IV, a detection limit of 1 fmol 8-oxodG is reported.
<table>
<thead>
<tr>
<th>Reaction</th>
<th>DNA Hydrolysis</th>
<th>Nucleotide 3’-monophosphates</th>
<th>Enrichment of 8-oxo-dG by HPLC fraction collection and drying</th>
<th>8-oxo-dG 3’-monophosphate</th>
<th>8-oxo-dG 3',5'-bisphosphate</th>
<th>Nuclease P1 removal of the 3’-p</th>
<th>Injection into HPLC</th>
<th>Quantitation using 32P-HPLC with on-line detection</th>
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</thead>
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<tr>
<td>Injection into HPLC</td>
<td>DNA hydrolysis using MN +SPD</td>
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5 THE PRESENT STUDY

5.1 AIM OF THE PRESENT INVESTIGATION

• Achieve a better understanding of the factors responsible for 8-oxodG formation, and try to reduce the formation of 8-oxodG during workup

• Study 8-oxodG formation in in vitro/in vivo models using free dG, DNA or cells, exposed to potential oxidants and antioxidants, and estimate background levels

• Improve the HPLC methodology for 8-oxodG analysis

• Evaluate 8-oxodG as a biomarker to measure oxidative stress

5.2 COMMENTS ON METHODOLOGY USED

The method used here (Papers II, IV, V) measures nucleosides from enzymatically hydrolysed nDNA, as mtDNA is removed during the initial nuclei collection steps by slow centrifugation after homogenisation.

5.2.1 Electrochemical detection

In Paper II, the HPLC system was optimised in terms of peak separation and stability of the EC-baseline at the point of time for 8-oxodG elution, within a relatively short time (< 20 min). Injection of pure 8-oxodG gave a detection limit as low as 5 fmol. The problems with EC detection are mainly due to baseline noise. HPLC-column washing using methanol resulted in a lowering of the baseline current and fewer baseline fluctuations (Paper VI). EC detection of 8-oxodG was found to be reproducible and accurate with few erroneous chromatograms. Removal of O₂ (g) from the HPLC-buffer by sparging with N₂ (g) or He (g) disturbed the EC-chromatogram severely (Paper II), and was not used. The amount of 8-oxodG and dG in DNA was determined using HPLC/EC/UV by comparison of the peak areas with calibration curves. Whereas the EC-curve is usually linear, the UV-curve can be slightly curved and fit better to a 2nd order equation.
5.2.2 $^{32}$P-HPLC

As shown in Paper I, incubations of dG at room temperature with varying amounts of $[^{32}\text{P}]\text{ATP}$ resulted in a linear, dose-dependent and very strong 8-oxodG formation. Based upon these data, a normal $^{32}$P-postlabelling procedure from the literature (50 µCi, 2 h) was calculated to give 19–25 additional 8-oxodG/10$^5$ dG, which is a substantial addition considering that normal levels are generally considered to be $\approx 0.1–1$ 8-oxodG/10$^5$ dG in control tissue. It was concluded that 8-oxodG should be labelled with $[^{32}\text{P}]\text{ATP}$ in the absence of dG, requiring a pre-separation step.

The pre-separation of 3'-monophosphate deoxynucleotides (dNps) before $^{32}$P-postlabelling of 8-oxodG was developed using HPLC separation (Papers III and IV). Analysing 1 µg amounts of calf thymus DNA gave a level of 0.52 ± 0.05 8-oxodG/10$^5$ dG. The detection limit was as low as 1 fmol. When comparing the $^{32}$P-HPLC method with HPLC/EC/UV using 2–10 µg DNA from human lymphocytes, a good correlation between the methods was seen (Paper IV). However, the levels of 8-oxodG increased considerably when analysing <5 µg for both methods, probably due to workup oxidation.

Figure 6. A representative HPLC chromatogram of hydrolysed DNA analysed by EC (upper) to detect 8-oxodG, and UV (lower) to detect dG.
of dG. For 20 µg DNA the [5'-\textsuperscript{32}P]8-oxo-pdG signal dropped, probably due to the presence of increasing amounts of normal nucleosides during the postlabelling step competing for [\textsuperscript{32}P]ATP. Increasing the amount of [\textsuperscript{32}P]ATP could solve this problem. The very sensitive \textsuperscript{32}P-HPLC method developed for 8-oxodG analysis offers the possibility of analysing small blood samples, needle biopsies and tissue swabs, but still needs further development in terms of reducing the workup oxidation, getting an even better separation of 8-oxodGp from other dNps, and increasing the reproducibility. The presented method is, however, considerably more laborious and time consuming compared to HPLC/EC/UV.

5.3 WAYS TO LIMIT WORKUP OXIDATION OF dG

When the DNA is taken out of a cell, the \textit{in vivo} protecting enzymes and antioxidants are no longer present to take care of the ROS. A low concentration of transition metals, peroxides and reducing agents are probably present in the solutions, which can oxidise dG with formation of 8-oxodG and false high levels as result. Also, 8-oxodG may be further oxidised into other products. Several methods can be used to limit the workup oxidation of dG, as discussed in Paper III with developments below.

\textbf{Removal of transition metals and reducing agents}

Use of high purity chemicals and enzymes, as well as Chelex treatment of buffers, is recommended in order to remove catalytic redox cycling transition metals. Storage of solutions can be done in plastic bottles (except sevag) to avoid glass release of transition metals. However, the biological sample being analysed will contain transition metals, or they may come from dust contamination of buffers. Further, enzymes used during workup may require metals for their function and, as DNA is a metal chelator, a zero concentration of transition metals in the solutions during workup is not realistic. Water of poor quality, Fe\textsuperscript{2+} and incubations with acid phosphatase (containing iron) were found to generate 8-oxodG (Paper II). Zn\textsuperscript{2+} which does not redox cycle was found necessary for efficient DNA hydrolysis using nuclease P\textsubscript{1} (Paper VI). Replacing protease with proteinase K lowered the 8-oxodG levels significantly, which may be due to contaminant transition metals in protease. The reducing agent GSH increased the artifactual 8-oxodG formation during workup (Paper VI) and ascorbate increased the 8-oxodG formation when incubated with free dG (Paper II).
Chelation of transition metals

The present study

The use of the Fe$^{3+}$-chelator desferal during the workup procedure can be successful since biological samples contain iron. However, the use of chelators may increase the catalytic role of other transition metals present, such as copper. It has been shown that EDTA-Fe$^{3+}$ is more catalytic in oxidising ascorbate compared to Fe$^{3+}$ alone, and that Cu$^{2+}$ is even more catalytic than EDTA-Fe$^{3+}$.[78] Desferal was more protective when excluding Mg$^{2+}$ and Ca$^{2+}$ from buffers, but was found to disturb the EC detector (Paper VI), which has also been reported.[155,156]

Oxidation of transition metals and reducing agents

Electron acceptors such as the nitrooxide TEMPO (free radical) can oxidise transition metals[157,158] and reducing agents[159] so that they cannot reduce peroxides, which in turn causes oxidation of dG. TEMPO was found to completely inhibit Fe$^{2+}$ (Paper II) and H$_2$O$_2$-ascorbate mediated (Paper V) 8-oxodG formation, but was incompatible with proteinase K, increasing the 8-oxodG formation (Paper VI). Nitroxides can also react by addition to alkyl radicals (R').[75]

Removal of peroxides from solutions

In Paper VI, GSH (reduced glutathione) generated artifactual formation of 8-oxodG during the workup procedure whereas H$_2$O$_2$ 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. H$_2$O$_2$ and other peroxides are likely to be present in the solutions to a small extent. Buffer components where peroxides can build up (often unsaturated carbon compounds) and storage conditions under light and heat should be avoided. Phenol, used to remove proteins during DNA extraction, may build up peroxides during storage. However, in Paper II high purity phenol or old pink phenol had no significant effect on 8-oxodG formation when incubated with free dG. Reports of significant artifactual 8-oxodG formation using phenol exist, however.[160,161] Long-term storage of 0.5% v/v Triton X-100 generated more 8-oxodG than Tween 20 when incubated with free dG (Paper VI), which is probably due to build-up of peroxides.[162,163] Catalase from Aspergillus niger was found effective as a remover of H$_2$O$_2$ without modifying the free dG molecule.
(Papers V and VI), whereas bovine catalase cleaved the free dG molecule (Papers I and V). Synthetic catalase mimics such as EUK-134[164] could also be tested.

**Fig 7.** Bovine catalase (50 U/mL) was found to cleave free dG (100 µM) when incubated at 37°C at pH 5.3.

**Reduction of temperature**

Lowered temperatures can drastically slow down unwanted reactions. The cold (0°C) high salt GTC (4 M guanidine thiocyanate) non-phenol DNA extraction method presented in Paper VI is likely to lead to less artifactual 8-oxodG formation than the 37°C RNase A/proteinase K methods normally employed. Freezing drastically slows down the collision of molecules. Storage of cells/tissue at -80°C was not found to modify the 8-oxodG content for several months. As shown in Paper II, 8-oxodG formation in pure buffer was found to be very strong at high temperatures (80–140°C). Also, at 80°C and above dG was hydrolysed, whereas 8-oxodG was not. Incubation on ice inhibited Fe²⁺-mediated 8-oxodG formation. In Paper V, temperature studies (-15 – +52°C) using free dG in the H₂O₂-ascorbate system showed that the least 8-oxodG was formed at low temperatures, but that 8-oxodG formation readily occurred at all tested temperatures. DNA hydrolysis using nuclease P₁ was found to be poor below 37°C (Paper VI).

**Fast purification and hydrolysis of DNA**

The quick (∼10 min) DNA isolation method using the cold (0°C) high salt GTC DNA extraction method (Paper VI) is likely to lead to less artifactual 8-oxodG formation than the 1.5–2.0 h, warm (37°C) RNase A/proteinase K methods normally employed. Also, concurrent hydrolysis using nuclease P₁ and alkaline phosphatase (Papers II and VI) is
The present study considerably faster than separate hydrolysis. The commonly used chaotropic NaI method\cite{104,155,165} is somewhat faster as it does not have a chloroform step to remove proteins, but is likely to co-precipitate proteinase K.

Increase the DNA concentration

As noticed in Paper IV and by several groups, small amount of cells/DNA give elevated 8-oxodG/dG ratios\cite{155,166,167}, and minimum amounts of > 20\cite{167}, 100\cite{155} µg DNA have been recommended. There are probably a certain number of oxidising agents, such as peroxides, present in a given volume. In Paper VI, 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: \( y = (8\text{oxodG}/10^5 \text{dG})_{\text{measured}}, a = (8\text{oxodG}/10^5 \text{dG})_{\text{background}}, b = 8\text{oxodG}_{\text{workup}} \) and \( x = \text{dG}_{\text{measured}} \). Thus, mathematically, at higher dG concentrations \( x \) the 8-oxodG formed during workup \( b \) will be “diluted out” and the level of 8-oxodG/10\(^5\) dG measured \( y \) will approach the true background level \( a \), so use of as much cells/DNA as possibly is recommended.

Removal of oxygen

The removal of O\(_2\) from the solutions will reduce the build-up of peroxides, but does not remove peroxides that are already present. Neither O\(_2\) itself nor O\(_2^-\) oxidises dG under normal conditions. In Paper II, argon sparging inhibited Fe\(^{2+}\)-mediated 8-oxodG formation, and O\(_2\) is likely to accept an electron from 8-OH-8-H-dG’ (see Figure 2). An anaerobic box with inert gas sparging of liquids was constructed for use during the workup procedure. However, performing a complete workup procedure for many samples inside the box was found to be very laborious, took a longer time and was more expensive (requiring a lot of nitrogen and argon gas). It was also difficult to keep the temperature low, as well as to fit various equipment in. Based on this, the anaerobic box was considered unsuitable for routine 8-oxodG analysis. DNA extraction under anaerobic conditions, however, has been found to lower the artifactual 8-oxodG formation.\cite{168}

Avoidance of energetic radiation

The radiation emitted from \(^{32}\)P or other unstable isotopes can directly ionise dG or give reactive species such as OH’ by radiolysis of water (Paper I). Energetic UV light or ionising radiation has long been known to generate H\(_2\)O\(_2\) in solutions,\cite{169} but effects of
background ionising radiation inside the lab are likely to be negligible during the workup procedure. Laboratory light had no significant effect on 8-oxodG formation (Paper II).

**Addition of a scavenger molecule for OH⁻**

The OH⁻ scavengers tested during experiments with Fe²⁺ (Paper II), H₂O₂-ascorbate (Paper V) and during workup (Paper VI), had only limited protective effects in inhibiting 8-oxodG formation. This suggests that free OH⁻ production was not the major mechanism responsible for 8-oxodG formation. Addition of OH⁻ scavengers, unless when added at extremely high concentrations, is not likely to have any great effect on 8-oxodG formation and may disturb enzymes, etc. In Paper V, a transition-metal mediated "two-electron reduction of H₂O₂" hydroxylation mechanism is suggested with one-electron oxidation of HAsc⁻ and dG respectively, producing the guanine radical cation (dG⁺⁺), which is hydroxylated by OH⁻/H₂O giving 8-oxodG. This is suggested to occur without formation of intermediate OH⁻ nor higher valence transition metals such as Fe(IV) or Cu³⁺. Changes in Gibbs free energy (ΔG⁰) calculations based on reduction potentials at pH 7 (E⁰) support a "two-electron reduction of H₂O₂" mechanism (also possible for other peroxides) over free, or "site specific"-produced OH⁻.

\[
\text{Fe(H₂O₂)²⁺ + dG} \rightarrow \text{Fe}^{3⁺} + 2\text{OH}⁻ + \text{dG}^{⁺⁺} \quad (29)
\]

\[
\text{OH}⁻ + \text{dG}^{⁺⁺} \rightarrow 8⁻ \text{OH} – 8⁻ \text{H} – \text{dG}⁻ \rightarrow 8⁻ – \text{oxodG} + \text{H}⁺ + \text{e}⁻ \quad (30)
\]

This mechanism takes into account the ease of oxidisability of the substrate, which OH⁻ production by one-electron reduction of H₂O₂ does not.

**Use of water substitutes**

As discussed in Paper VI, isotopically labelled H₂¹⁸O should generate 8-(¹⁸O)oxodG during workup which can be separated from the normal 8-(¹⁶O)oxodG using mass spectrometry methods. Methanol, ethanol, DMSO etc. were found not to dissolve DNA and to inhibit enzymatic DNA hydrolysis.

**Others**

There was less formation of 8-oxodG at low pH (Papers II and V), although 8-oxodG formation still occurred.
Vacuum- and freeze-drying have been suggested as oxidising steps.[149]

Cutting of RNA and proteins could generate oxidant species capable of oxidising dG, although no such results have yet been reported. Formation of $^1\text{O}_2$ during workup is unlikely, but cannot be excluded.
6 CONCLUSIONS

• HPLC/EC/UV is a very sensitive, reproducible and accurate system for detection of 8-oxodG and dG

• $^{32}$P was found to strongly oxidise dG, and for $^{32}$P-postlabelling of 8-oxodG the pre-separation of 8-oxodG from dG was developed using HPLC

• Peroxides and iron are involved in the artifactual generation of 8-oxodG during the workup procedure

• Several inhibitory steps can be employed to reduce artifactual generation of 8-oxodG from dG during workup. A fast ($\approx$10 min) cold (0°C) DNA extraction procedure was developed, and use of catalase and TEMPO were found to be protective. However, effects of added "antioxidants" during workup and analysis should be tested for, as they may mediate artifactual 8-oxodG formation, modify nucleosides or disturb the chromatographic system or detector

• A new hydroxylation mechanism of carbon compounds by peroxides is suggested, where transition metals mediate the two-electron reduction of $\text{H}_2\text{O}_2$, with one-electron oxidation of the reducing agent and compound respectively, producing carbon radical cations that are hydroxylated in water. For dG oxidation in a $\text{H}_2\text{O}_2$-ascorbate system, this mechanism is shown to be more thermodynamically favourable than a one-electron reduction of $\text{H}_2\text{O}_2$, which produces OH$^-$ and, further, the addition of OH$^-$ scavengers was found to be ineffective. Peroxide oxidation of DNA is likely, if forming DNA radical cations, to give mainly oxidation of G due to electron transfer in DNA, compared to systems generating the more randomly adding OH$^-$ (such as $\gamma$-radiolysis of water)

• Oxidation of dG to 8-oxodG during workup was found to be relatively constant for a certain method and to fit a mathematically defined curve which can help in estimating the true background level and the artifactual formation of 8-oxodG

• Background 8-oxodG/dG ratios in human lymphocytes are much lower than previously reported

• To obtain more reliable results, as the degree of workup formation of 8-oxodG can differ for different days of workup, it is recommended that control samples are included during the workup procedure, having an equal amount of cells (or DNA) as the exposed samples

• Some results need to be re-examined due to the problems with workup formation of 8-oxodG. Despite this, 8-oxodG can be considered to be a good biomarker for oxidative stress, based on published results
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