Reaction of HO• with Guanine Derivatives in Aqueous Solution: Formation of Two Different Redox-Active OH-Adduct Radicals and Their Unimolecular Transformation Reactions. Properties of G(-H)•

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Abstract: The reaction of •OH with 2′-deoxyguanosine yields two transient species, both identified as OH adducts (G•-OH), with strongly different reactivity towards O_2, or other oxidants, or to reductants. One of these, identified as the OH adduct at the C-8 position (yield 17% relative to •OH), reacts with oxygen with $k = 4 \times 10^9\,\text{M}^{-1}\,\text{s}^{-1}$; in the absence of oxygen it undergoes a rapid ring-opening reaction ($k = 2 \times 10^5\,\text{s}^{-1}$ at pH 4–9), visible as an increase of absorbance at 300–310 nm. This OH adduct and its ring-opened successor are one-electron reductants towards, for example, methylviologen or $[Fe^{III}(CN)_6]^{3-}$. The second adduct, identified as the OH adduct at the 4-position (yield of 60–70% relative to •OH), has oxidizing properties (towards *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, promethazine, or $[Fe^{II}(CN)_6]^{4-}$). This OH adduct undergoes a slower transformation reaction ($k = 6 \times 10^3\,\text{s}^{-1}$ in neutral, unbuffered solution) to produce the even more strongly oxidizing (deprotonated, depending on pH) 2′-deoxyguanosine radical cation, and it practically does not react with oxygen ($k \leq 10^6\,\text{M}^{-1}\,\text{s}^{-1}$). The (deprotonated) radical cation, in dilute aqueous solution, does not give rise to 8-oxoguanosine as a product. However, it is able to react with ribose with $k \leq 4 \times 10^3\,\text{M}^{-1}\,\text{s}^{-1}$.

Keywords: DNA oxidation · electron transfer · kinetics · radicals

Introduction

Hydroxyl radical (HO•) oxidation or modification of nucleic acids has been and still is the subject of very active research. As a physiological and endogenous oxidant, this radical is central to many health/disease phenomena and, of course, it is of great interest in radiological or toxicological situations. The most sensitive target for HO• is the nuclear material of the cell. Numerous pathways for hydroxyl radical oxidation of DNA exist.[1–4] In general, the nucleobases are more reactive than the sugar-phosphate moiety by a factor of 5–10,[5] and each purine or pyrimidine forms a characteristic series of modified derivatives.[6–8] Many of the products of these pathways, for example, 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 8-oxoguanine, have become diagnostic for the intermediacy of HO•.[8–11] However, in order to quantify their significance, it is necessary to not only understand the *initial* reactions of HO• with the nucleic acid bases, but also the rapid follow-up reactions of the radicals produced on their way to the final, non-radical product(s), which are usually deleterious to the functioning of DNA. Concerning oxidative damage, guanine (G) is the most sensitive of the DNA bases.[12–19] The simplest conceivable oxidation reaction is an electron transfer, which would give rise to the radical cation of G, that is, $G^{\bullet+}$. There is evidence that among the various oxidizing agents that may lead to $G^{\bullet+}$, HO• is in fact involved. However, the evidence is also that HO• leads to other products as well.[2, 3, 11, 13, 17, 20] A more thorough understanding of the pattern of reaction of HO• with G is therefore necessary, and an attempt at this is described in the following.

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Results and Discussion

Formation and transformation reactions of the OH adducts: The hydroxyl radical reacts with guanine and its derivatives in neutral aqueous solution with diffusion-limited rates ($\approx 5 \times 10^9\,\text{M}^{-1}\,\text{s}^{-1}$).[5] As an example, in Figure 1 are shown the time-resolved absorption spectra measured on reaction of the hydroxyl radical with 1 mM 2′-deoxyguanosine (≡dGuo) in an N_2O-saturated, unbuffered solution at pH 7.6. The spectra observed are characterized by absorptions in the wavelength range ≈280 nm to ≈750 nm; the rapid increase of absorbance (see insets), which was complete in ≤1 μs after the 300 ns pulse, is due to *formation* of the "OH adduct(s)" (see later). Subsequent to the formation of the OH adduct(s) the spectrum changed: there was a *build-up* of absorbance in

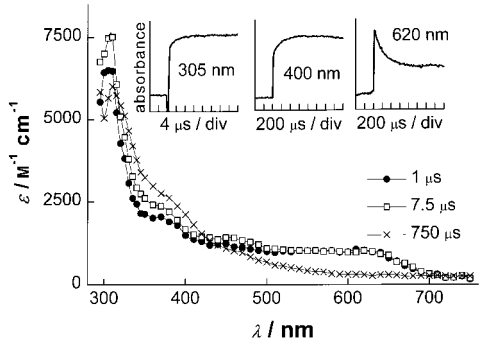

Figure 1. Transient absorption spectra observed on reaction of the hydroxyl radical with 2′-deoxyguanosine (1 mM), by pulse radiolysis in N_2O-saturated, unbuffered solution at pH 7.6. The spectra were recorded at the times after the pulse indicated in the figure. The inserts show the absorbance changes at the wavelengths indicated.

the 300–310 nm range and at ≈400 nm, but a *decay* at ≈620 nm (see insets to Figure 1). These processes follow first-order kinetics with rates independent of the concentration of the parent compound (but dependent on pH, temperature, and phosphate and oxygen concentration, see Figure 2). In unbuffered solution at pH 7.6 the rate constants of these transformations are $2.7 \times 10^5\ s^{-1}$ for the build-up at 305 nm, and $5 \times 10^3\ s^{-1}$ for both the build-up at 400 nm and the decay at 620 nm. With the other guanine derivatives (guanosine, guanosine-5′-phosphate, 2′-deoxyguanosine-5′-phosphate, 1-methylguanosine and 9-methylguanine) similar spectral transformations were observed.

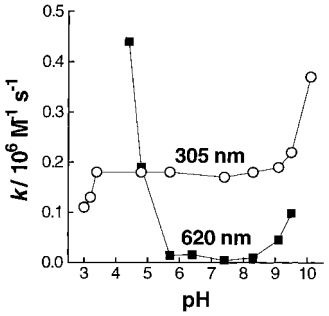

Figure 2. pH dependence of the rate constants of the transformation reactions of OH adducts of 2′-deoxyguanosine (build-up at 305 nm and decay at 620 nm, as indicated) observed by pulse radiolysis of N_2O-saturated, unbuffered, aqueous solutions.

The two different rates (2.7×10^5 and $5 \times 10^3\ s^{-1}$) measured for the different spectral changes suggest that (at least) two different OH adducts are formed that undergo different transformation reactions.

The rates of the spectral transformations were found to be pH dependent. For example, for dGuo, the (first-order) rate of build-up at 305 nm, which was invariant with pH between 4 and 9, decreased at pH < 4, whereas at pH > 9 it increased. In comparison, the decay rates at 620 nm and the build-up at 400 nm were invariant with pH in the range 6–8.5 ($k \approx 6 \times 10^3\ s^{-1}$). In neutral, unbuffered solution these processes were slow, but higher concentrations of H^+ and OH^- accelerated the reaction (Figure 2).

Also phosphate accelerated the build-up at 400 nm and the decay at 620 nm, and in a similar manner. At pH 7, the rate of these processes increased linearly with increasing phosphate concentration (up to 5 mM); the slope of this dependence yielded the rate constant for phosphate "catalysis" at pH 7, $k = 6.7 \times 10^7\ M^{-1}\ s^{-1}$. The phosphate effect was found to be pH dependent: in the pH range 5–9, for which the H^+/OH^- catalysis is negligible, the rate measured in the presence of 1 mM phosphate decreased with increasing pH following a sigmoidal curve with an inflection point at pH 6.9, which is that of the $H_2PO_4^-/HPO_4^{2-}$ equilibrium. This result indicates that $H_2PO_4^-$ is a more efficient catalyst of the reaction than its conjugate base HPO_4^-; their catalytic rate constants are $1.8 \times 10^8\ M^{-1}\ s^{-1}$ and $3.5 \times 10^7\ M^{-1}\ s^{-1}$, respectively. In comparison with the spectral changes at 400 and 620 nm, the rate of build-up at 305 nm at pH 7 was *not* affected by phosphate up to a concentration of 20 mM. This again shows the different nature of the radicals responsible.

In summary, the species formed by reaction of $^{\bullet}OH$ with dGuo and derivatives each undergo a first-order transformation reaction that have different rates and which are additionally distinguishable by the effect that pH and phosphate have on their rates. One reaction, visible as an increase of absorbance at ≈305 nm is accelerated by OH^-, inhibited by H^+, and unaffected by phosphate at pH 7. A second transformation, visible as a decay at ≈620 nm and a build-up at ≈400 nm, is enhanced by H^+, OH^-, and phosphate.

In order to further characterize these reactions, the effect of temperature on their rates in neutral, unbuffered solution was investigated. Both processes followed good Arrhenius behavior in the temperature range 0 to 60 °C. As with the pH or phosphate effect, the increase at 400 nm and the decay at 620 nm exhibited identical behavior, which was different from that of the build-up at 300–310 nm. The activation parameters were measured for the different processes and are given in Table 1. As evident, their different rates at room temperature are due mainly to the difference in activation *entropy* of the processes.

The rates of the transformation reactions of the OH adducts of dGuo, which both have low activation enthalpy and negative activation entropy (Table 1), were also determined in unbuffered D_2O. The build-up at 300–310 nm shows the isotope effect $k_{H2O}/k_{D2O} = 2.5$, which is essentially due to an increase in activation enthalpy (which overrides the effect of

Table 1. Activation parameters and isotope effects on the transformation reactions of the HO-Adducts of 2′-deoxyguanosine in unbuffered, neutral solution.

	$\Delta H^{\neq}$ [kJ mol^{-1}]	$\Delta S^{\neq}$ [J mol^{-1} K^{-1}]	k_{H2O}[a] [s^{-1}]	k_{H2O}/k_{D2O}[a]	$\Delta H^{\neq}_{H2O}/\Delta H^{\neq}_{D2O}$	$\Delta S^{\neq}_{H2O}/\Delta S^{\neq}_{D2O}$
build-up 310 nm	18.5	−77	2.7×10^5	2.5	0.8	1.2
decay 620 nm	17.3	−115	5×10^3	0.7_5	1.3	0.9

[a] At 20 °C.

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the increasing activation entropy in this direction) on changing the solvent from H_2O to D_2O. In contrast, the decay at 620 nm exhibits $k_{H2O}/k_{D2O} = 0.75$, which essentially results from a lower activation enthalpy in D_2O, relative to H_2O. Taking all these observations together, it is thus clear that in the reaction of $OH^{\bullet}$ with dGuo two different radicals are produced.

An additional confirmation for this is the effect of oxygen. This was investigated taking solutions of dGuo saturated with a 4:1 (v:v) mixture of N_2O (to scavenge e^-_{aq}) and O_2 (to react with radicals). Under these conditions, the initial spectrum of the OH adducts was the same as in oxygen-free solution. Furthermore, the decay of absorbance at 620 nm and the build-up at 400 nm were not affected by the presence of oxygen. In an experiment with guanosine in unbuffered, neutral solution, the concentration of oxygen was taken to saturation (≈ 1.4 mM). The decay at 620 nm could still be observed and had the same rate ($6 \times 10^3\ s^{-1}$) as in oxygen free solution. From these results it is evident that the precursor species of this transformation does not react rapidly with oxygen ($k \leq 10^6\ M^{-1}s^{-1}$). This in agreement with earlier observations.[21, 22]

In contrast, the increase of absorbance in the range 300–310 nm was quenched by oxygen (Figure 3) and, as a result, the amplitude of the absorbance at 300 nm, after the completion of the transformation reaction (≈ 20 μs after the pulse), decreased with increasing oxygen concentration.

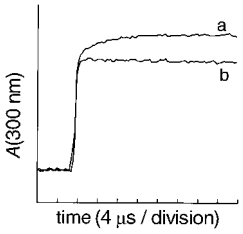
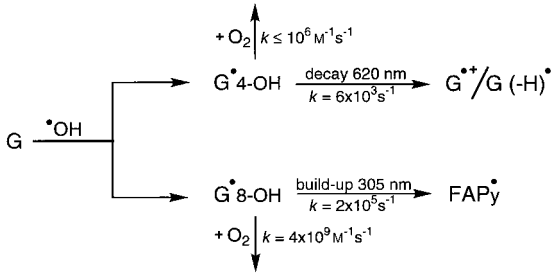


Figure 3. Transient absorption at 300 nm observed on reaction of $^{\bullet}OH$ with 2′-deoxyguanosine in solution saturated with a) N_2O, or b) with a N_2O:O_2 gas mixture (4:1 v:v).

These results can be explained (see Scheme 1) by the competition between the transformation reaction (build-up at 300–310 nm) of the OH adduct (G$^{\bullet}$8-OH, see later) and its reaction with oxygen. From the rate of reaction measured in the absence of oxygen ($k = 2 \times 10^5\ s^{-1}$) and the dependence of the absorbance at 300 nm on the oxygen concentration, the rate of reaction of G$^{\bullet}$8-OH with oxygen can be estimated as $k \approx 4 \times 10^9\ M^{-1}s^{-1}$, that is, diffusion controlled.



Scheme 1.

The second adduct, which undergoes a slower transformation reaction ($k = 6 \times 10^3\ s^{-1}$) in neutral, unbuffered solution, is identified in terms of the adduct with the OH at the 4-position and is termed G$^{\bullet}$4-OH. It is oxidizing in nature (see later), and does not react rapidly with oxygen, as shown above.

Redox titration[13] of the OH adducts

Oxidizing radicals

Reaction with reductants: It has been previously shown[21, 23–26] that the addition of hydroxyl radicals to some heterocyclic compounds generates isomeric radicals that can be distinguished on the basis of their redox properties. Along these lines, in order to test for the formation of *oxidizing* radicals, we have now investigated the reaction of the OH adducts of dGuo and derivatives with the *reductants* promethazine (PZ^+) and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD). Upon oxidation, both these compounds give rise to persistent radicals with strong absorbance bands in the visible region. For example, the promethazine *radical* dication ($PZ^{\bullet 2+}$) has a reduction potential $E(PZ^{\bullet 2+}/PZ^+) = 0.92$ V versus a standard hydrogen electrode (SHE), which is pH independent in the range pH 0–9; it absorbs in the visible with $\lambda_{max} = 510$ nm and $\varepsilon = 7980\ M^{-1}cm^{-1}$.

Pulse radiolysis experiments were performed with N_2O-saturated solutions of guanosine, 2′-deoxyguanosine, or 9-methylguanine (2–5 mM in each case) in 5 mM phosphate at pH 7 and which contained promethazine in variable concentration up to 0.5 mM. Under these conditions, on the basis of the rate constants,[5] it is the *guanine derivatives* that capture most of the $^{\bullet}OH$ formed by irradiation, yielding the respective OH adducts (Scheme 1). However, the experimental result is that it is $PZ^{\bullet 2+}$ that is formed, as shown by an increase of absorbance at 510 nm with rate proportional to the PZ^+ concentration (Figure 4). This is due to the HO adducts of the guanine derivatives G$^{\bullet}$4-OH (see later) oxidizing PZ^+ to yield $PZ^{\bullet 2+}$; the rate constant of this reaction, determined from the dependence of the observed rate of $PZ^{\bullet 2+}$ formation on $[PZ^+]$ is $2 \times 10^9\ M^{-1}s^{-1}$ for all the guanine derivatives used in these experiments.

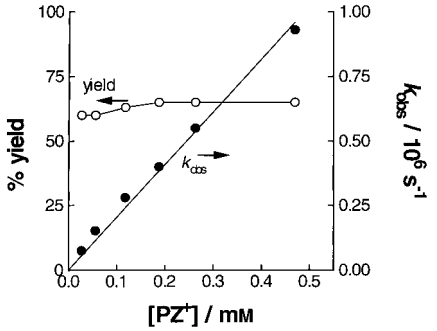


Figure 4. Observed rate (solid symbols) and yield (relative to the total yield of radicals, open symbols) of formation of promethazine radical dication (monitored at 510 nm) formed on reaction of promethazine (PZ^+) with the species resulting from reaction of the hydroxyl radical with 2′-deoxyguanosine (2 mM) in N_2O-saturated, phosphate buffer (5.7 mM) at pH 7.

The bimolecular rate *constant* did not change when the *observed rate* was lower or higher than the transformation reactions (see later) of the oxidizing G•-OH radicals, G4•-OH. This means that promethazine reacts with the precursors G4•-OH and products ($G^{\bullet+}$/G(-H)• see Scheme 1 and later) of this reaction with equal rates. The *yield* of $PZ^{\bullet 2+}$ was determined from the absorbance at 510 nm after completion of its build-up. The results show that despite the rapid reaction of the radicals from guanine and derivatives with PZ^{+}, the yield of $PZ^{\bullet 2+}$ accounts for only ≈65% of the total yield of radicals (Figure 4). Thus, the yield of oxidizing radical, G•4-OH, is ≈65% of the OH adducts formed whilst the remaining ≈35% are not oxidizing.

Similar results were obtained with TMPD. The respective radical cation ($TMPD^{\bullet+}$) has the (low) reduction potential $E(TMPD^{\bullet+}/TMPD) = 0.27$ V versus SHE, independent of the pH in the range 8–14,[27] and absorption in the visible region, $\lambda_{max} = 565$ nm $\varepsilon = 12{,}500\,M^{-1}cm^{-1}$.[23] The radicals formed on reaction of •OH with guanosine, 2′-deoxyguanosine, or 9-methylguanosine at pH 8 reacted with TMPD with rate constants $\approx 2 \times 10^9\,M^{-1}s^{-1}$ to yield $TMPD^{\bullet+}$ in ≈65% yield, that is, the same number as in the case with PZ^{+}. Thus, from the experiments with TMPD or PZ^{+}, it is clear that the reaction of •OH with these guanine derivatives yields ≈65% oxidizing radicals (identified in terms of G•4-OH), a number similar to that (70%) from the conductance experiments (see later), which measured the yield of radical cation.[28]

Also the weaker (relative to TMPD) and, therefore, presumably more selective reductants hexacyanoferrate(II) ($E([Fe^{III}(CN)_6]^{3-}/[Fe^{II}(CN)_6]^{4-}) = 0.36$ V vs. SHE) and hexachloroiridate(III) ($E([Ir^{IV}Cl_6]^{2-}/[Ir^{III}Cl_6]^{3-}) = 0.86$ V vs. SHE) were treated with the radicals generated on reaction of •OH with dGuo. In the *absence* of reductants in N_2O-saturated solution, buffered at pH 7 by 2 mM phosphate, the transformation reaction seen as a decay at 620 nm had the rate constant $1.5 \times 10^5\,s^{-1}$ (Figure 5a). When up to 0.1 mM $K_4[Fe^{II}(CN)_6]$ was added to the solution, this reaction was still visible, but a second, slower component appeared that brought the absorbance back down to the pre-pulse value (Figure 5b). The rate of this second component increased with increasing concentration of $[Fe^{II}(CN)_6]^{4-}$. At 420 nm, where ($[Fe^{III}(CN)_6]^{3-}$) absorbs, a *build-up* of absorbance was observed (not shown) that had the same rate as the slow component of the decay at 620 nm. From this it is concluded that the reaction leads to the formation of $[Fe^{III}(CN)_6]^{3-}$, that is, that a radical able to oxidize $[Fe^{II}(CN)_6]^{4-}$ is present. For concentrations of $[Fe(CN)_6]^{4-} > 0.1$ mM the decay at 620 nm became mono-exponential, with the rate still dependent on the concentration of $[Fe(CN)_6]^{4-}$. However, the *slopes* of the straight lines describing the observed rate of decay at 620 nm as a function of concentration $[Fe(CN)_6]^{4-}$ were different (Figure 5c) for $[Fe(CN)_6]^{4-}$ concentrations < 0.1 mM (slow component, $k_1([Fe(CN)_6]^{4-}) = 3.2 \times 10^9\,M^{-1}s^{-1}$), and for concentrations $[Fe(CN)_6]^{4-} > 0.1$ mM ($k_2([Fe(CN)_6]^{4-}) = 1.8 \times 10^9\,M^{-1}s^{-1}$).

This "biphasic" behavior was more evident with the still weaker reductant $[Ir^{III}Cl_6]^{3-}$. In this case, the transformation reaction of the HO adducts of dGuo was monitored at 650 nm (Figure 6a), where neither $[Ir^{III}Cl_6]^{3-}$ nor $[Ir^{IV}Cl_6]^{2-}$ absorb. For concentrations of reductant < 0.6 mM, the decay at 650 nm showed two components (Figure 6a): the fast component was unaffected by $[IrCl_6]^{3-}$, but the slow component, which brought the absorbance back to zero (Figure 6b), had a rate dependent on the concentration of $[IrCl_6]^{3-}$ (Figure 6d). At 490 nm, where the iridium^{IV} complex absorbs, an *increase* of absorbance was observed (Figure 6c); this had the same rate as the slow component of the decay at 650 nm. The concentration of hexachloroiridate(III) was further increased up to 2.3 mM and the absorbance at 650 nm immediately after the pulse became smaller; this can be attributed to the competition[5] between dGuo and hexachloroiridate(III) for the hydroxyl radicals. At concentrations > 1.5 mM the rate of decay at 650 nm became mono-exponential and the rate did not increase with further increase of the concentration of hexachloroiridate(III) (Figure 6d).

The biphasic behavior of the reaction between the dGuo radicals and $[Fe(CN)_6]^{4-}$ or $[IrCl_6]^{3-}$ can be explained by competition between the unimolecular transformation reaction G•4-OH → $G^{\bullet+}$/G(-H)•, seen as a decay of absorbance at 620 nm, and the bimolecular reaction of G•4-OH or $G^{\bullet+}$/G(-H)• with the reductants (Scheme 2).

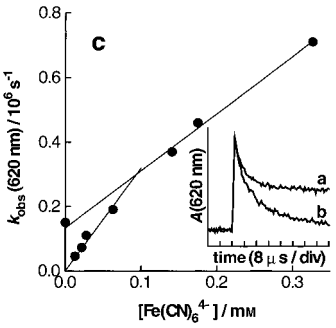


Figure 5. Transient absorbance at 620 nm measured on reaction of hydroxyl radicals with 2′-deoxyguanosine (1 mM) in N_2O-saturated phosphate buffer (2 mM) at pH 7, a) in the absence and b) in the presence of hexacyanoferrate(II) 13 μM. c) Observed rate of decay at 620 nm as a function of the hexacyanoferrate(II) concentration.

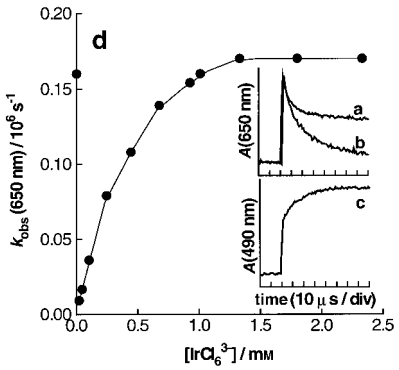


Figure 6. Transient absorbance measured on reaction of hydroxyl radicals with 2′-deoxyguanosine (same conditions as in Figure 5a), a) without hexachloroiridate(III) at 650 nm and b) with hexachloroiridate(III) at 650 nm and c) at 490 nm. d) Observed rate of decay at 650 nm as a function of the concentration of hexachloroiridate(III).

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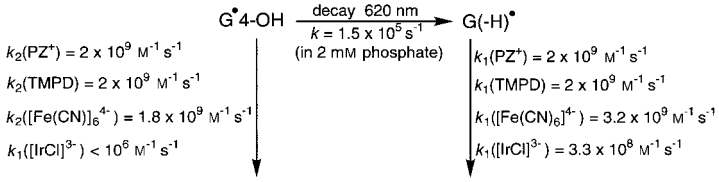


Scheme 2.

For explanation of Scheme 2, at low concentrations of hexacyanoferrate(II) or hexachloroiridate(III), the (unimolecular) transformation reaction is faster than the (second-order) reaction of G•4-OH with the reductant. In contrast, at higher concentrations of hexacyanoferrate(II) or hexachloroiridate(III) (>0.1 mM or >1 mM, respectively), it is the transformation reaction that becomes rate limiting: In the case of $IrCl_6^{3-}$, the observed rate of reaction is found not to increase further. In contrast, with the stronger reductant hexacyanoferrate(II), the observed rate *does* increase further with increasing concentration, albeit with a slope lower than at lower concentrations of $Fe(CN)_6^{2-}$ (see Figure 5). This means that $Fe(CN)_6^{3-}$, a stronger reductant than $IrCl_6^{3-}$, is able to reduce both G•4-OH and its transformation product $G^{\bullet+}$ (or G(-H)•, depending on pH), although the former with a lower rate, whereas the weaker reductant $IrCl_6^{3-}$ is able to react only with the stronger oxidant $G^{\bullet+}$ or G(-H)•. The organic reductants promethazine and TMPD are not selective and react with G•4-OH and $G^{\bullet+}$ with indistinguishable rates.[29]

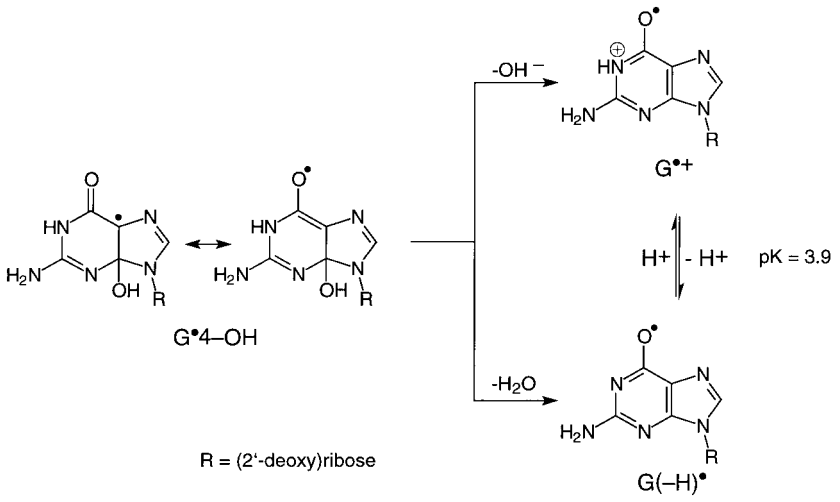
Thus, as a preliminary conclusion, from the experiments with PZ^+ and TMPD it is evident that $\approx 65\%$ of the radicals formed on reaction of •OH with the guanine derivatives have oxidizing properties (identified as G•4-OH, see Scheme 3 later). Furthermore, on the basis of the results obtained with $Fe(CN)_6^{4-}$ and $IrCl_6^{3-}$, the transformation reaction of the OH adducts detected as a decay of absorbance at 620 nm is assigned to the conversion of the oxidizing G•4-OH to the (more strongly) oxidizing radical $G^{\bullet+}$/G(-H)•. The guanine radical cation and its conjugate base are in fact strong oxidants as shown by their reduction potentials, for example, $E_7(G(-H)^\bullet,H^+/G) = 1.29$ V[19].

The reactivity of dGuo(-H)• with O_2 was studied by observing its effect on the decay of dGuo(-H)• in a solution containing 1.5 mM deoxyguanosine and 0.1 M KBr at pH 7. From the minimal effect seen, $k(dGuo(-H)^\bullet + O_2)$ is calculated to be $\leq 10^6\,M^{-1}s^{-1}$. However, when the conditions were chosen such that the radical $O_2^{\bullet-}$ was additionally produced, there was a faster decay of dGuo(-H)•. From this effect the rate constant for the reaction $dGuo(-H)^\bullet + O_2^{\bullet-}$, which *could* proceed by addition, is estimated to be $3 \times 10^9\,M^{-1}s^{-1}$.[30] In an analogous experiment the reactivity of dGuo(-H)• with the radical $Me_3C^\bullet$, produced by the reaction of •OH with di-*tert*-butylsulfoxide,[31] was tested. Also in this case the reactivity found is large, $\approx 10^9\,M^{-1}s^{-1}$.

Conductance changes involving the oxidizing radicals: We have previously used conductance detection to study the reaction of the sulfate ($SO_4^{\bullet-}$) or dibromine ($Br_2^{\bullet-}$) radicals with guanosine, dGuo, and 1-methylguanosine.[32] It was found that these reactions lead to the formation of the radical cations, $G^{\bullet+}$. Furthermore, the radical cations were found to deprotonate to yield the neutral radical G(-H)•, with $pK_a = 3.9$ for (2′-deoxy)guanosine and $pK_a = 4.7$ for 1-methylguanosine. Now, in order to see if OH• is also able to produce radical cations, conductance detection was again applied. It was previously shown[33] that the reaction of •OH with guanosine does not lead to changes in conductance in neutral solution, but in acid solution a decrease of conductance is observed that follows a p*K* curve with inflection point at pH = 3.9. The decrease is due to replacement of the mobile H^+ by the less-conducting radical cation of guanosine. We have confirmed these results and, in addition for the first time, quantified at pH 3 the *yield* of decrease of conductance. On the basis of the results obtained with guanosine, 2′-deoxyguanosine, and 1-methylguanosine, H^+ is consumed (= the radical cation produced) with a yield of 70% relative to the •OH radicals generated by the radiation pulse, a value essentially the same as that of the oxidizing radicals (see previous section).[34]

Identification of G•4-OH: In the case of adenine derivatives it has been demonstrated[25, 35, 36] that the adducts at the 4-position have oxidizing properties. By analogy and since the OH adduct at the 4-position of guanine derivatives, G•4-OH, should have considerable unpaired spin density at the electrophilic oxygen at C6, as shown below, we identify the oxidizing OH adduct as G•4-OH. As argued above, this adduct undergoes water elimination to yield $G^{\bullet+}$/G(-H)• (Scheme 3).

The assignment of water elimination from an OH adduct is consistent with the acid–base catalysis observed for this reaction. The rate enhancement by H^+ is due to protonation



Scheme 3.

of the (potential) OH^- leaving group giving rise to the much better leaving group H_2O. Bases can also catalyze these reactions, as deprotonation at another position assists the OH^- elimination.[37] Also the inverse isotope effect observed for this reaction is consistent with an acid–base equilibrium preceding the rate-determining step [Eq. (1)].

$$G^{\bullet}\text{-}OH + H_2O\ (D_2O) \rightleftharpoons G^{\bullet}\text{-}OH_2^+\ (G^{\bullet}\text{-}OHD^+) + OH^-(OD^-) \qquad (1)$$

D_2O, which is a stronger acid than H_2O,[38] shifts the equilibrium [Eq. (1)] to the right increasing the observed rate of water elimination.

Reducing radicals

Reaction with oxidants: Radicals formed on reaction of $^{\bullet}OH$ with the guanine derivatives were treated with the oxidants hexacyanoferrate(III) or *N*,*N*′-dimethyl-4,4′-bipyridinium (methylviologen, MV^{2+}). A solution of guanosine in N_2O-saturated, neutral solution containing hexacyanoferrate(III) may serve as an example. The absorbance at 420 nm was found to decay with a rate proportional to the concentration of hexacyanoferrate(III) added, yielding the second-order rate constant $k = 1.7 \times 10^9\,M^{-1}s^{-1}$ for this process. The decrease of absorbance at 420 nm is indicative of the depletion of hexacyanoferrate(III); it suggests that $Fe(CN)_6^{3-}$ reacted with a transient species formed on reaction of $^{\bullet}OH$ with guanosine, probably being reduced to $Fe(CN)_6^{4-}$, which does not absorb at 420 nm. Since the yield of this reaction could not be determined accurately from the change of absorbance, as the extinction coefficient of $Fe(CN)_6^{3-}$ ($\varepsilon_{420\,nm} = 1020\,M^{-1}cm^{-1}$) is of the same order as that of the guanosine radicals (cf. Figure 1), conductometric detection (AC) was used. The experiments were performed at pH 5, which is above both the pK_a of the guanosine radical cation (p$K_a = 3.9$),[32] and the pK for the protonation of $Fe(CN)_6^{4-}$ (p$K = 4.2$).[39] For calibration, the results were compared with those obtained on radiolysis of similar solutions containing isopropanol (25 mM) instead of guanosine. Under these conditions, there is an increase of conductance, which is attributed to the reduction of $Fe(CN)_6^{3-}$ by radicals from isopropanol with formation of H^+ [Eq. (2)].

$$^{\bullet}C(CH_3)_2OH + Fe(CN)_6^{3-} \longrightarrow (CH_3)_2C{=}O + Fe(CN)_6^{4-} + H^+ \qquad (2)$$

Also in the guanosine solutions an increase of the conductance of the solution was observed; this consisted of a fast component, within the dead-time of the conductance detection (2–3 μs), and a slower, exponential component. The *rate* of the latter was proportional to the concentration of hexacyanoferrate(III), with a second-order rate constant $k = 1.5 \times 10^9\,M^{-1}s^{-1}$ (Figure 7). This number is similar to the $1.7 \times 10^9\,M^{-1}s^{-1}$ measured optically (see above). Also the *yield* of H^+ was dependent on the concentration of hexacyanoferrate(III) (Figure 7). When the concentration was increased up to 0.21 mM, the yield of H^+ was found to tend to a constant value of ≈35% of the total yield of radicals, divided into ≈15% in the slow component, and the remaining ≈20% in the fast component.

The second oxidant used was methylviologen (MV^{2+}). Reduction of MV^{2+} [$E(MV^{2+}/MV^{\bullet+}) = -0.45$ V vs. SHE]

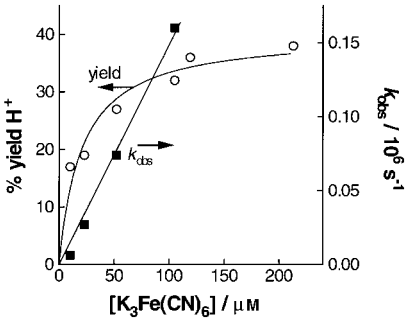


Figure 7. Rates of increase of conductance (solid symbols) and yields of H^+ relative to the total yield of radicals (open symbols), measured on reaction of hexacyanoferrate(III) with the guanosine radicals formed by reaction with $^{\bullet}OH$ in N_2O-saturated, unbuffered solution at pH 5.

generates the long-lived radical cation $MV^{\bullet+}$ with an intense absorption in the visible region ($\lambda_{max} = 605$ nm, $\varepsilon = 14\,500\,M^{-1}cm^{-1}$). Pulse radiolysis experiments were performed with N_2O-saturated, neutral solutions of guanosine (1 mM) containing MV^{2+} (up to 3 mM). A build-up of absorbance at 605 nm was observed, which was larger than in the solutions that did not contain MV^{2+}. This build-up exhibited a fast ($k_{obs} \geq 10^6\,s^{-1}$) and a slow component. This suggests that at least two different species reactive towards MV^{2+} are formed on reaction of $^{\bullet}OH$ with guanosine (see later). The rate of the slow component increased linearly with the concentration of methylviologen (Figure 8); from the slope of the linear dependence, the rate constant is $k = 1.4 \times 10^8\,M^{-1}s^{-1}$. However, the extrapolation to zero concentration yielded the intercept $k = 2 \times 10^5\,s^{-1}$. The yield of the *slow* component tended to a value of ≈13% of the total yield of radicals.

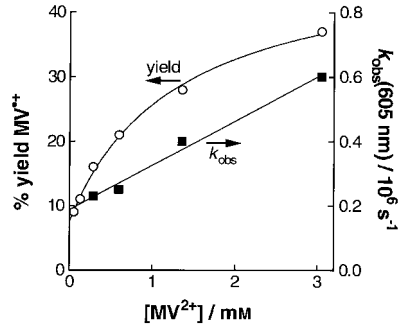


Figure 8. Rates of formation (solid symbols) and yields of methylviologen radical cation (relative to the total yield of radicals, open symbols), monitored by the increase of absorbance at 605 nm on reaction of methylviologen with the guanosine radicals formed by reaction with $^{\bullet}OH$ in N_2O-saturated phosphate buffer (1 mM) at pH 5.

The yield of the *fast* component increased with increasing methylviologen concentration, up to 3 mM. From a plot of the reciprocal yield of the fast component against the reciprocal of the methylviologen concentration, we estimate the yield of the fast component as ≈17% of the total yield of radicals.

The observations with the oxidants $[Fe(CN_6)]^{3-}$ and MV^{2+} can be explained by the competition between reactions with these oxidants of a reducing radical and the unimolecular rearrangement of the radical. The reducing radical is identified in terms of the OH adduct at the 8-position, G8-OH, in

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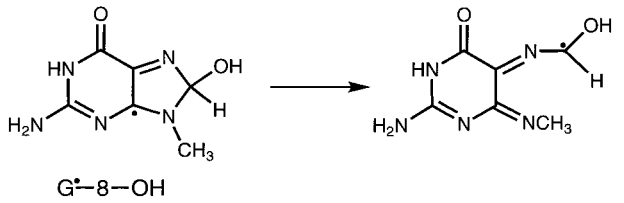
analogy to reactions of adenine and derivatives[25, 35, 36] in which it was found that the HO adduct at the 8-position reacts with oxidants, including oxygen, in competition with the scission of the C8–N9 bond, which leads to opening of the imidazole ring. Applied to the guanine system, a similar scheme ressults (Scheme 4), with the fast transformation reaction of the OH adduct (build-up of absorbance at 300–310 nm) being assigned to the ring-opening reaction. On the basis of the reactivity with methylviologen and hexacyanoferrate(III), it can be concluded that at least two species with reducing properties are formed on reaction of the hydroxyl radical with guanosine, in yields ≈13–15% and 17–20%, respectively.



Scheme 4.

In order to obtain information on the nature of the two types of reducing radicals, experiments were performed with 9-methylguanine as a model for (deoxy)guanosine. The radicals formed on reaction of 9-methylguanine with •OH also reacted with methylviologen and hexacyanoferrate(III). However, the build-up of absorbance at 605 nm in the presence of methylviologen, as well as the increase of conductance in the presence of hexacyanoferrate(III) at pH 5, exhibited only one fast component; the slow component observed with guanosine was missing. From this it may be concluded that the slow component, with a yield of 13%, is due to the reaction of methylviologen or hexacyanoferrate(III) with radicals produced by H abstraction from the (deoxy)-ribose moiety, whereas the fast component, with a yield of 17%, is due to G•8-OH.

Product analysis: To complement the results obtained with pulse radiolysis, experiments were performed with 9-methylguanine (9-MeG) as a model with the aim of quantifying the depletion of the parent compound and the yield of the product oxygenated at the 8-position (8-hydroxy-9-methylguanine 8-HO-9-MeG, or more correctly 8-oxo-7,8-dihydro-9-methylguanine). Solutions of 9-MeG in N_2O-saturated phosphate buffer (1 mM) at pH 7 were irradiated with doses up to 60 Gy, and the concentration of parent compound was determined by HPLC with optical and electrochemical detection. The concentration of 9-MeG decreased linearly with increasing absorbed dose; the depletion of parent compound relative to the total hydroxyl radical formed was, however, only 15%. Similar experiments were carried out in the presence of hexacyanoferrate(III) (0.1 mM) or oxygen (0.27 mM). The depletion of substrate was increased to 18% by oxygen and to 30% by hexacyanoferrate(III) (all figures relative to the total •OH generated).

Under the conditions of these experiments, the hydroxyl radical generated by the radiation reacts quantitatively with 9-MeG. In spite of this, the depletion of 9-MeG is incomplete. This indicates the operation of efficient repair processes by which radicals are converted back to the parent compound.

Also the yield of 8-HO-9-MeG[40] formed on reaction of •OH with 9-methylguanine was measured. It was found to depend on the pH and the presence of additives. For example, at pH 4, the yield of 8-HO-9-MeG was 8%; increasing the concentration of hexacyanoferrate(III) increased the yield until for concentrations of oxidant > 0.2 mM the yield became constant at ≈20%. Also at pH 7, hexacyanoferrate(III) increased the yield of 8-HO-9-MeG, but only at small concentration (< 10 μM); further increase of the concentration of oxidant *decreased* the yield of the 8-oxygenated product (Figure 9a). Control experiments showed that the decrease of yield is not due to decomposition of the 8-HO-9-MeG by hexacyanoferrate(III).

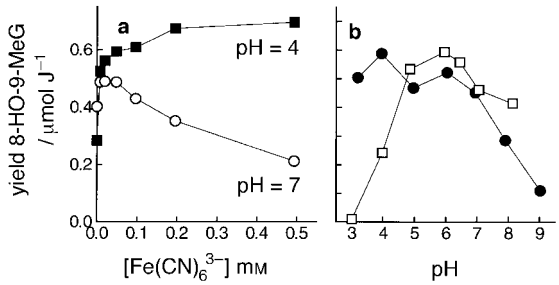
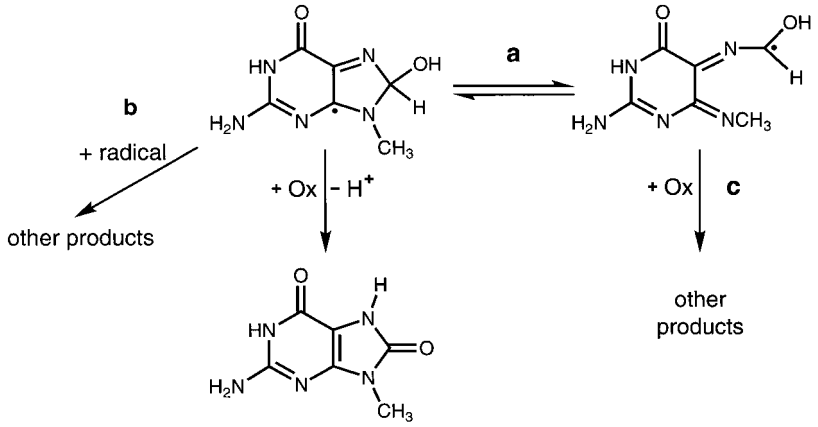


Figure 9. Yields of 8-oxo-7,8-dehydro-9-methylguanine (8-hydroxy-9-methylguanine 8-HO-9-MeG) formed on reaction of hydroxyl radical with 9-methylguanine (100 μM) in N_2O-saturated phosphate buffer (1 mM). a) In the presence of different concentrations of hexacyanoferrate(III) at pH 4 and 7, as indicated. b) At different pH in the absence (open squares) and in the presence (solid circles) of hexacyanoferrate(III) 50 μM.

The formation of the product oxygenated at the 8-position and the *increase* of its yield in the presence of oxidants can be rationalized by the addition of •OH to the 8-position, followed by oxidation of the adduct in competition with the opening of the imidazole ring, followed by reactions of the ring-opened radicals. However, this simple scheme does not explain the experimental observations fully. Firstly, the concentration of oxidant required to reach the maximum yield of 8-HO-9-MeG (at pH 7) is surprisingly low: on the basis of the rate of the transformation reaction of the reducing radical (assigned to the ring-opening reaction, $k = 2.7 \times 10^5\ s^{-1}$), the concentration of oxidant required to reach the maximum concentration of 8-HO-9-MeG would have to be of the order ≈1 mM, even if the reaction of G•8-OH with the oxidant was limited by diffusion. Secondly, a *decrease* of the yield of 8-HO-9-MeG with increasing oxidant concentration is not consistent with a simple competition between ring-opening and oxidation of G•-8-OH.

The experimental observations can, however, be explained by a mechanism involving a *reversible* ring-opening reaction (Scheme 5, step a). According to this hypothesis, low concentrations of oxidant increase the yield of the 8-oxygenated product by competing with the bimolecular decay of radicals (step b). As the concentration of oxidant is increased, the oxidation of the ring-*opened* radical (step c) starts to domi-



Scheme 5.

nate preventing the ring-reclosure and this leads to a *decreased* yield of the product oxygenated at the 8-position.

Also the pH dependence of the yield of 8-HO-9-MeG (Figure 9b) is rather complex. In the absence of oxidant, the yield is at a maximum at neutral pH, but decreases rapidly at either higher or lower pH. In contrast, in the presence of oxidant, the yield of 8-HO-9-MeG is approximately constant between pH 3 and 7, but decreases in alkaline solution. These observations can, in principle, be understood in terms of acid–base catalysis on the ring-opening reaction of 8-HO-9-MeG$^{\bullet}$.

An alternative mechanism for the formation of products oxygenated at the 8-position would be the hydroxylation at C8 of the guanine radical cation, or its conjugate base, by water addition followed by deprotonation of the H_2O adduct. This reaction would give rise to the 8-HO-G$^{\bullet}$-type radical whose oxidation would yield the 8-HO-G derivative. In order to investigate this possibility, a solution of guanosine (1 mM) and potassium bromide (0.1 M) in N_2O-saturated phosphate buffer (1 mM) at pH 7 was γ-irradiated with a dose of 400 Gy. Under these conditions, the dibromide radical is formed, and reacts rapidly with guanosine by electron transfer to yield exclusively the deprotonated guanosine radical cation, G(-H)$^{\bullet}$.[32] We have now found that under these conditions no 8-hydroxyguanosine (8-HO-Guo) is formed. Also at pH 3, at which the oxidation of guanosine by dibromide radical yields the radical cation G$^{\bullet+}$, no formation of 8-HO-Guo could be detected. Equally, in the presence of hexacyanoferrate(III) (to oxidize the hypothetical 8-OH adduct formed by water addition to the radical cation) at either pH 3 or pH 7 no 8-HO-Guo was formed. Similar experiments were performed with guanosine solutions containing thallium(I) (1 mM) instead of bromide. Irradiation of these solutions generates Tl^{II}, which oxidizes guanosine to the radical cation G$^{\bullet+}$ or its conjugate base G(-H)$^{\bullet}$.[41] Again, no 8-HO-Guo could be detected. The conclusion is thus that the rate of the hypothetical water addition to G$^{\bullet+}$ or G(-H)$^{\bullet}$ is much smaller than the bimolecular decay of these radicals. Under the γ-radiolysis conditions used, the lifetime of these is 1–10 s.[42] Another possibility is that 8-HO-G is formed from the radical cation by reaction with O_2. To test this idea, the radical cation was produced at pH 3 or 7 by reaction with Tl^{II} or $Br_2^{\bullet-}$ as described above, but in the presence of 1 mM O_2. Again, no 8-OH-G was found. These results show that in dilute aqueous solution hydration of the (deprotonated) radical cation, if it occurs, is either very slow ($k < 0.1\ s^{-1}$) or it does not lead to 8-OH-G$^{\bullet}$.[43]

However, 8-OH-G is clearly formed on reaction of G with the *hydroxyl radical*. Also, 8-OH-G has been found as a product of the 193 nm photolysis of Guo.[44] The fact that 8-OH-G is *not* produced from G$^{\bullet+}$/G(-H)$^{\bullet}$, as pointed out above, means that its formation by the 193 nm light is *not by a photoionization pathway*. This indicates that the photolysis-induced strand breakage reported for DNA[45, 46] is not necessarily related to photoionization of the nucleic acid bases (and thus to the formation of G$^{\bullet+}$/G(-H)$^{\bullet}$). It may, instead, be due to a so-far unidentified non-ionic photochemical reaction.

Reaction of guanosine radicals with ribose: As shown above, more than half of the radicals generated on reaction of the guanine derivatives with the hydroxyl radical undergo water elimination to yield, depending on pH, the radical cation or its conjugate base, G(-H)$^{\bullet}$ (Scheme 3). The same radical is formed when ionization of DNA generates a positive charge, which is able to migrate over a considerable distance[47–49] to become trapped probably at guanine,[12–19, 50], more specifically, at a GG doublet or GGG triplet.[51–54] It is of interest to investigate whether this radical can lead to strand breaks, the lethal type of DNA damage. To intitiate a strand break,[55] "radical transfer" from the base to a deoxyribose moiety is necessary. In order to test whether this type of reaction is at all possible, the guanosine radical G(-H)$^{\bullet}$ was generated by reaction of $Br_2^{\bullet-}$ with guanosine at pH 7. The absorbance of $Br_2^{\bullet-}$ at 360 nm was monitored. A fast build-up of absorbance was observed, followed by an exponential decay. This is due to the formation of $Br_2^{\bullet-}$ and its decay by reaction with guanosine leading to G(-H)$^{\bullet}$. After this reaction, the absorbance, now solely due to G(-H)$^{\bullet}$, decayed very slowly (on the timescale of milliseconds, see Figure 10a). Addition of D-ribose up to 50 mM led to an acceleration of the decay of G(-H)$^{\bullet}$ (Figure 10b).

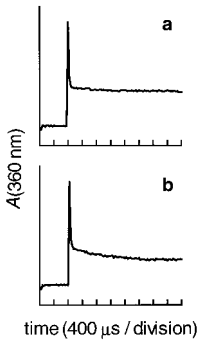


Figure 10. Transient absorption at 360 nm observed on pulse radiolysis of guanosine solutions (1 mM) in N_2O-saturated phosphate buffer (1 mM) at pH 7 containing 0.1 M potassium bromide, a) in the absence and b) in the presence of 50 mM D-ribose.

From the dependence of this effect on the ribose concentration an estimate was made on the rate of reaction between G(-H)$^{\bullet}$ and ribose: $k \leq 4 \times 10^3\ M^{-1}s^{-1}$.[56] If this reaction, which can reasonably only be

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an H abstraction from ribose, takes place (in *uni*molecular fashion) in the DNA polymer, a ribose-centered radical (which could be the C4′-radical) will be formed that will lead[57, 58] to a strand break. Strand breaks have in fact been explained[45] as originating from G•+/G(-H)•.

Conclusions

The reaction of •OH with dGuo yields two transient species, identified as the OH adducts at the 4- and 8-position, G•4-OH and G•8-OH, with yields of 65–70% and 17%, respectively.[59] They have strongly different reactivity towards O_2, other oxidants, or to reductants. G•8-OH reacts with oxygen with a diffusion-controlled rate; in the absence of oxygen it undergoes a rapid ring-opening reaction ($k = 2 \times 10^5\ s^{-1}$ at pH 4–9) that is visible as an increase of absorbance at 300–310 nm. G•8-OH is a one-electron reductant towards, for example, methylviologen or $[Fe^{III}(CN)_6]^{3-}$. In contrast, G•4-OH has oxidizing properties (towards *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, promethazine, or $[Fe^{II}(CN)_6]^{4-}$). G•4-OH undergoes a slower transformation reaction ($k = 6 \times 10^3\ s^{-1}$ in neutral, unbuffered solution) to produce the more strongly oxidizing (deprotonated, depending on pH) dGuo radical cation, and it practically does not react with oxygen ($k \leq 10^6\ M^{-1}s^{-1}$). The (deprotonated) radical cation, in dilute aqueous solution, oxygenated or not, does not give rise to 8-OH-Guo. However, it is able to react with ribose ($k \leq 4 \times 10^3\ M^{-1}s^{-1}$). The (deprotonated) radical cation, G(-H)•, reacts with $O_2^{\bullet-}$ or with *tert*-butyl radicals with high rates.

Experimental Section

All chemicals were of the highest quality commercially available. The pulse radiolysis experiments were performed as previously described.[23, 32] For product analysis, the samples were exposed to the γ-radiation from a ^{60}Co source; the dose rate was typically about 0.4 $Gy\,s^{-1}$ and the total dose was such that <40% of the starting material was converted. The products of irradiation were separated and quantified by HPLC, with Nucleosil-C_{18} columns. The mobile phase was an aqueous solution of KH_2PO_4 (2 mM), $NaClO_4$ (20 mM), and methanol (4%) at pH 4. The flow-rate was such that complete elution was achieved in about 15 min. Simultaneous electrochemical ($E = +0.7$ V vs. Ag/AgCl) and UV ($\lambda = 254$ or 293 nm) detection was performed. Resorcinol was used as internal standard and as calibration of the electrochemical detection.

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[29] These results are in line with observations reported previously (P. O'Neill, P. W. Chapman, *Int. J. Radiat. Biol.* **1985**, *47*, 71) that the oxidizing radical(s) formed on reaction of •OH with dGuo have the same reactivity as the radical formed from the one-electron oxidation of dGuo with $Br_2^{\bullet-}$ towards a series of medium to strong reductants. This oxidizing radical is now identified as the radical cation or its conjugate base, that is, G•+ or G(-H)•, which is formed through the elimination of OH^- or H_2O from the oxidizing G•4-OH (see Scheme 2 or 3).

[30] In order to explain the experimentally observed formation of imidazolone and oxazolone as final oxidation products of the guanine moiety, the reaction of G(-H)• with O_2 has been proposed as the initial step (J. Cadet, M. Berger, G. W. Buchko, P. C. Joshi, S. Raoul, J. L. Ravanat, *J. Am. Chem. Soc.* **1994**, *116*, 7403; S. Raoul, M. Berger, G. W. Buchko, P. C. Joshi, B. Morin, M. Weinfeld, J. Cadet, *J. Chem. Soc. Perkin Trans. 2* **1996**, 371), but this has not been observed by pulse raiolysis. Now, a kinetically more favored alternative to this can be put forward: addition of $O_2^{\bullet-}$ to G(-H)• followed by protonation of the adduct to give the hydroperoxide and its subsequent decomposition by the "Cadet-mechanism" (see above).

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[42] It is interesting that 8-hydroxy-derivatives of dGuo were also not formed on *photochemical* production (by sensitization by riboflavin) of the radical cation of dGuo *in dilute aqueous solution of dGuo*, in

contrast to when the dGuo radical cation was generated in *ds DNA*, in which 8-HOdGuo was produced in high yield (see H. Kasai, Z. Yamaizumi, M. Berger, J. Cadet, *J. Am. Chem. Soc.* **1992**, *114*, 9692). This can be explained by assuming a much longer lifetime of the radical cation in DNA (K. Hildenbrand, D. Schulte-Frohlinde, *Free Radical Res. Commun.* **1990**, *11*, 195, have measured the lifetime of the deprotonated dGuo radical cation in ds DNA in aqueous solution to be 5 s, while in ss DNA its lifetime was too short to be measured) compared with the monomeric dGuo, such that only in ds DNA the very slow hydration reaction has a chance to proceed (an alternative is that the hydration reaction is much faster in ds DNA than in the case of the "monomeric" radical cation). For further discussion, see A. Spassky, D. Angelov, *Biochemistry* **1997**, *36*, 6571 and D. Angelov, A. Spassky, M. Berger, J. Cadet, *J. Am. Chem. Soc.* **1997**, *119*, 11373.

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