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## Special Issue Invited Review

# Biphotonic Ionization of DNA: From Model Studies to Cell†

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## ABSTRACT

**Oxidation reactions triggered by low-intensity UV photons represent a minor contribution with respect to the overwhelming pyrimidine base dimerization in both isolated and cellular DNA. The situation is totally different when DNA is exposed to high-intensity UVC radiation under conditions where biphotonic ionization of the four main purine and pyrimidine bases becomes predominant at the expense of singlet excitation processes. The present review article provides a critical survey of the main chemical reactions of the base radical cations thus generated by one-electron oxidation of nucleic acids in model systems and cells. These include oxidation of the bases with the predominant formation of 8-oxo-7,8-dihydroguanine as the result of preferential hole transfer to guanine bases that act as sinks in isolated and cellular DNA. In addition to hydration, other nucleophilic addition reactions involving the guanine radical cation give rise to intra- and interstrand cross-links together with DNA–protein cross-links. Information is provided on the utilization of high-intensity UV laser pulses as molecular biology tools for studying DNA conformational features, nucleic acid–protein interactions and nucleic acid reactivity through DNA–protein cross-links and DNA footprinting experiments.**

## INTRODUCTION

Solar irradiation of cells and skin predominantly gives rise to the formation of three main classes of bipyrimidine photoproducts in a strong sequence-dependent manner as the result of the direct excitation of thymine and cytosine bases (1–3). These products include the overwhelming cyclobutane pyrimidine dimers (CPDs) together with the less abundant pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) that may be subsequently converted by UVA photons into the corresponding Dewar valence isomers (2,3). UVB radiation has been shown to be a very poor oxidizing agent as illustrated by the low yield of 8-oxo-7,8-dihydroguanine (8-oxoG), an ubiquitous DNA oxidation product, which represents <1% of CPDs observed in UVB-irradiated AS52 CHO cells (4). In addition, DNA single-strand breaks (SSBs), alkali-labile sites (ALS) and oxidized pyrimidine bases are also generated with a very low efficiency mostly as the result of hydroxyl radical ($^{\cdot}OH$) reactions. It is likely that $^{\cdot}OH$ participates in the UVB-induced formation of 8-oxoG together with a much smaller contribution of guanine photoionization as postulated earlier (2) from the observed susceptibility of purine bases to UVC radiation (5,6). This recently received confirmation from the measurement of 8-oxoG and spermine–guanine adducts in calf thymus DNA upon either UVC or UVB irradiation (7). Interestingly, three adjacent guanines in the human telomere G-quadruplex sequence were found to be 10-fold more susceptible to monophotonic ionization than in double-stranded DNA with a relative yield of 8-oxoG/CPDs close to 1:30 (8). It is expected from the well-documented chemistry of guanine radical cation (9,10) that 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 2,2,4-triamino-5(2*H*)-oxazolone (Oz) could also be generated as two other minor UVB-induced DNA photoproducts.

UVA irradiation of isolated and cellular DNA leads to the predominant formation of CPDs (11–13) as a result of the direct excitation of pyrimidine bases (14). Under these irradiation conditions, however, 6-4PPs are not generated in detectable yields in contrast to the formation of oxidatively produced DNA damage that is significantly increased. Thus, 8-oxoGua is preferentially generated over strand breaks and oxidized pyrimidine bases mostly from photosensitized reactions (2,11). The formation of 8-oxoG in cellular DNA has been rationalized in terms of a predominant type II photosensitization pathway (2,3,15) giving rise to singlet oxygen ($^1O_2$), a reactive oxygen species (ROS) that is able to diffuse up to a 150–220 nm distance inside cells (16) before reacting with vulnerable targets such as guanine. Other oxidative degradation pathways that give rise to SSBs and oxidized pyrimidine bases are triggered by $^{\cdot}OH$ (11,17,18), the most oxidizing ROS generated by the one-electron reduction of hydrogen peroxide ($H_2O_2$), the dismutation product of unreactive superoxide anion radical ($O_2^{\cdot-}$) as an indirect contribution of type I photosensitization reactions (15).

Formation of the guanine radical cation ($G^{\cdot+}$) via type I photosensitization is rather unlikely in cellular DNA. This may be

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explained by the inability of most endogenous and exogenous type I photosensitizers to be in close contact with DNA bases, which is a prerequisite condition to promote efficient electron transfer. However, type I photosensitizers have been used in model studies involving nucleosides and isolated DNA to decipher the chemical reactions of purine and pyrimidine radical cations (19–22) as further discussed below. A suitable strategy based on type I photosensitized generation of a proximate base radical cation in defined sequence duplex DNA fragments has been designed to investigate the mechanisms of hole migration along oligonucleotide chains (23,24).

One of the most convenient systems to efficiently generate radical cations in cellular DNA consists in exposing biological samples to high-intensity UVC (266 nm) nanosecond laser pulses (25). This has been found to promote biphotonic ionization of the nucleobases (Fig. 1) as already established more than 30 years ago in pioneering studies (26–28). This photochemical approach has also been shown to be a relevant tool for the efficient generation of DNA–protein cross-links (DPCs) (29,30) and for footprinting studies of DNA secondary structure (31), applications that will be also reviewed. It may be pointed out that an alternative way to trigger the formation of guanine radical cations into oligonucleotides is based on the selective biphotonic ionization of embedded 2-aminopurine using intense UVB (308 nm) excimer nanosecond laser pulses (32,33).

### Pyrimidine nucleosides

*Thymidine (Thd).* Thd radical cations ($Thd^{\cdot+}$) are induced upon exposure to ionizing radiation, vacuum UV and high-intensity UVC laser pulses (25,34,35). In addition, several Type I photosensitizers (2-methyl-1,4-naphthoquinone, riboflavin, nitro derivatives of lysine) are able to generate $Thd^{\cdot+}$ in aqueous solution by electron transfer from the pyrimidine to excited photosensitizers mostly in their triplet state. There are two main decomposition pathways of pyrimidine radical cations, in general, which include hydration of the radical cation site and deprotonation from a nearby acidic position. In the case of $Thd^{\cdot+}$, hydration and deprotonation (Fig. 2) represent approximately 60% and 40%, respectively (36–40). The hydration pathway involves the specific addition of $H_2O$ to the C6 position of Thd radical cations giving initial 6-hydroxy-5,6-dihydrothymid-5-yl radicals (41) that transform in part upon $O_2$ addition into the corresponding *cis* and *trans* diastereomers of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (38,39). In addition, a number of stable products are also formed resulting from the decomposition of intermediate peroxyl radicals and hydroperoxides, including the four *cis* and *trans* diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine (ThdGly), the 5*R** and 5*S** forms of 5-hydroxy-5-methylhydantoin and *N*-formamide nucleoside derivatives. The deprotonation pathway of $Thd^{\cdot+}$ involves initial loss of a proton from the methyl group giving rise to the allylic 5-methyl-(2′-deoxyuridylyl) radical (38,39). The degree of deprotonation is sensitive to pH and the $pK_a$ of the photosensitizer radical anion. The presence of a methyl group at N3 increases the formation of products arising from the hydration pathway in a particular case, suggesting that deprotonation also takes place from the N3 position of the $Thd^{\cdot+}$ (42). In the absence of oxygen, the 5-methyl-(2′-deoxyuridylyl) radical undergoes either reduction or oxidation depending on the redox properties of additional compounds (43,44). In aqueous aerated solutions, $O_2$ efficiently competes for the 5-methyl-(2′-deoxyuridylyl) radical leading to the corresponding peroxyl radical. The methylperoxyl radical is subsequently reduced to the relatively stable 5-(hydroperoxymethyl)-2′-deoxyuridine by a two-step process involving reduction (*i.e.* with $O_2^{\cdot-}$) and protonation or a one-step process via H-atom abstraction (*i.e.* thiols). In addition, the methylperoxyl radical undergoes bimolecular decay giving alcohol and aldehyde consisting of 5-(hydroxymethyl)-2′-deoxyuridine (5-HmUrd) and 5-formyl-2′-deoxyuridine (5-FodUrd) respectively according to a Russel mechanism. Although relative stable in ultrapure aqueous solutions, 5-(hydroperoxymethyl)-2′-deoxyuridine is sensitive to thermal and metal-catalyzed decomposition. Therefore, 5-(hydroperoxymethyl)-2′-deoxyuridine undergoes dehydration to

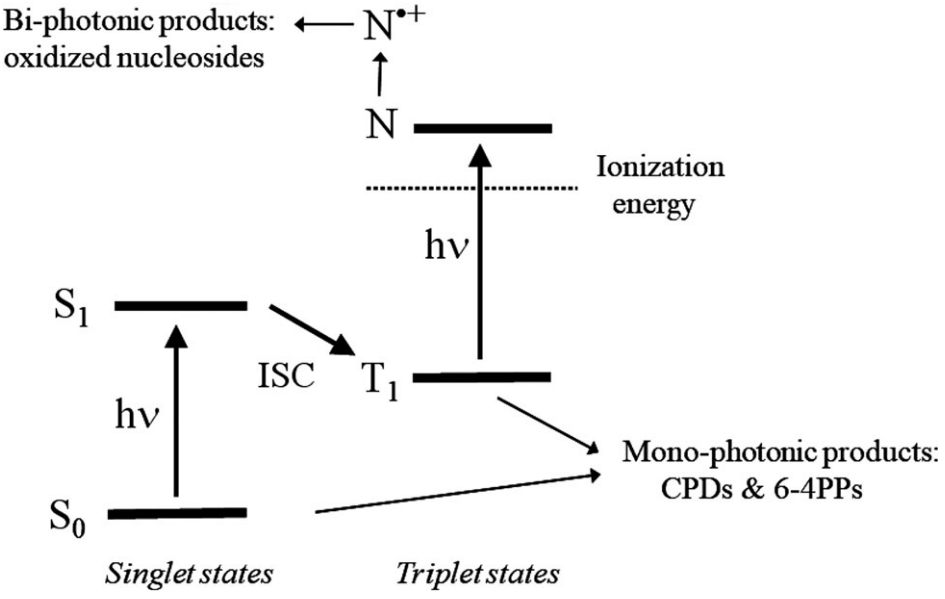


**Figure 1.** Monophotonic and biphotonic initial mechanisms of UVC nanosecond laser-induced DNA damage. A triplet excited state is implicated as an intermediate transient in the biphotonic ionization of pyrimidine bases, whereas for the purine bases, it is not possible at the present stage to rule out the participation of a long-lived singlet excited manifold.

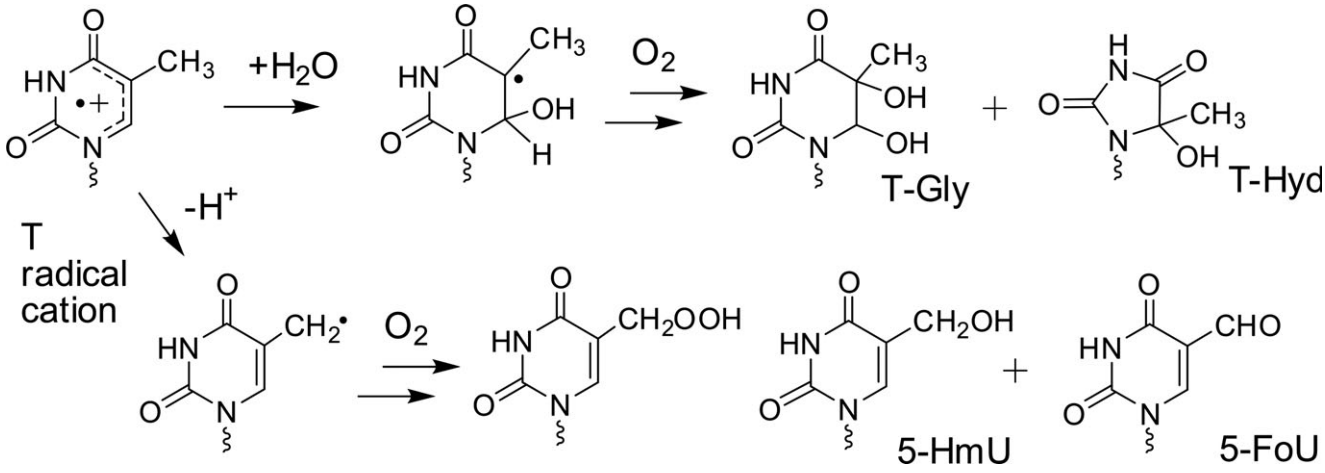


**Figure 2.** Conversion pathways of thymine radical cation through either hydration or deprotonation in aerated aqueous solution.

5-FodUrd, while reduction of the hydroperoxide by trace metal ions gives 5-HmdUrd (45). The ratio of 5-HmdUrd and 5-FodUrd ultimately depends on the concentration of $O_2$ and the presence of other redox agents in the solution. There is evidence today that the 5-methyl-(2′-deoxyuridylyl) radical in DNA can abstract a hydrogen from a neighboring thymine base leading to contiguous base damage (46,47).

*2′-Deoxycytidine (dCyd).* The radical cation of dCyd ($dCyd^{\cdot+}$) is efficiently generated in aqueous solution by near-UV photosensitization with 2-methyl-1,4-naphthoquinone (40). On the basis of product analyses, the mechanism of formation of stable products arising from $dCyd^{\cdot+}$ is divided into two competitive pathways of hydration and deprotonation (Fig. 3). Hydration of $dCyd^{\cdot+}$ leads to both hydroxyl-substituted 5,6-dihydroxy-2′-deoxycytidyl radicals, which subsequently transform into a complex mixture of stable and unstable products, including dCyd 5,6-glycols, imidazolidine, isodialuric acid, dialuric acid and 5-hydroxyhydantoin products (48,49). The lack of substitution at C5 (H instead of $CH_3$) of dCyd favors loss of $H_2O$ or $H_2O_2$ from intermediate 5,6-glycols and 5,6-hydroxyhydroperoxides to 5-hydroxy-2′-deoxycytidine (5-OHdCyd). In the case of saturation of the 5,6-pyrimidine bond, the ensuing unstable products (*i.e.* cytosine glycols) are subject to efficient deamination into the corresponding uracil derivatives (products with a carbonyl rather than an amino group at C4). The other main transformation pathway of $dCyd^{\cdot+}$, deprotonation, involves the loss of a proton from one of the two predominant acidic sites of dCyd radical cations. The formation of 2′-deoxyuridine (dUrd) as a major product of MQ-photosensitized oxidation of dCyd (48) indicates that deprotonation of $dCyd^{\cdot+}$ occurs at the exocyclic amino group. Upon deprotonation, the initial neutral N6 aminyl radical of cytosine is stabilized by formation of the iminyl sigma-radical in which N3 is protonated as determined by ESR and DFT analyses (50). The intermediate formation of $N^4$-aminyl radicals has also been confirmed by ESR measurements in the photosensitization of 1-methylcytosine by triplet anthraquinone-2,6-disulfonic acid (51). In addition, $dCyd^{\cdot+}$ undergoes deprotonation from the C1′ position of the sugar moiety leading to the release of cytosine and 2-deoxyribono-1,4-lactone in equal amounts (52). There are several differences in the chemistry of Thd and dCyd radical cations. Firstly, the hydration of $dCyd^{\cdot+}$ occurs at both C5 and C6 of the pyrimidine ring in a ratio of about 1 to 2 based on [$^{18}$O]-labeling experiments (48). Secondly, the 5,6-hydroxyhydroperoxides of dCyd are not stable in aqueous solution although they have been tentatively identified from the sonolysis of dCyd in aqueous oxygenated solutions (53). Thirdly, deprotonation of $dCyd^{\cdot+}$ occurs at both the exocyclic amino group and the C1′ anomeric position of the nucleoside.

*5-Methyl-2′-deoxycytidine (5-MedCyd).* 5-MedCyd is more susceptible to one-electron oxidation than either Thd or dCyd. Indeed, the formation of 5-MedCyd radical cations ($5\text{-}MedCyd^{\cdot+}$) as monitored by electron magnetic resonance was favored upon irradiation of crystals of cytosine, even though 5-methylcytosine was only present in trace amounts (54,55). $5\text{-}MedCyd^{\cdot+}$ was recently characterized by its IR absorption spectra following fs two-photon ionization; the rate of deprotonation was estimated to be in the ns range, similar to the radical cation of dGuo (56). The deprotonation of $5\text{-}MedCyd^{\cdot+}$ gives 5-methyl-(2′-deoxycytidylyl) radicals (Fig. 3) as the major reaction of the nucleoside in aqueous solution (~60%) (57). The methyl-centered radical likely transforms into the corresponding peroxyl radical and hydroperoxide (half-life = 9.5 h at 24°C). In turn, the hydroperoxide decomposes into stable methyl oxidation products namely 5-(hydroxymethyl)-2′-deoxycytidine and 5-formyl-2′-deoxycytidine. The case of 5-MedCyd radical cations involves the same reactions observed for Thd and dCyd radical cations with certain exceptions. In particular, the intermediate products that arise from hydration of the 5,6-double bond of 5-MedCyd are less prone to deamination than those of dCyd. In addition, the extent of deprotonation increased from 40% for Thd radical cation to 60% for $5\text{-}MedCyd^{\cdot+}$ and the latter occurs predominantly at the C5 methyl group.

## Purine nucleosides

*2′-Deoxyguanosine (dGuo).* As observed for pyrimidines, the main fate of purine nucleoside radical cations in aqueous solution involves hydration and deprotonation (Fig. 4). Hydration of dGuo radical cation ($dGuo^{\cdot+}$) at C8 gives rise to 8-hydroxy-7,8-dihydroguanyl radicals, which undergo, in a competitive manner, oxidation (*e.g.* in the presence of $O_2$) to give 8-oxodGuo or reduction

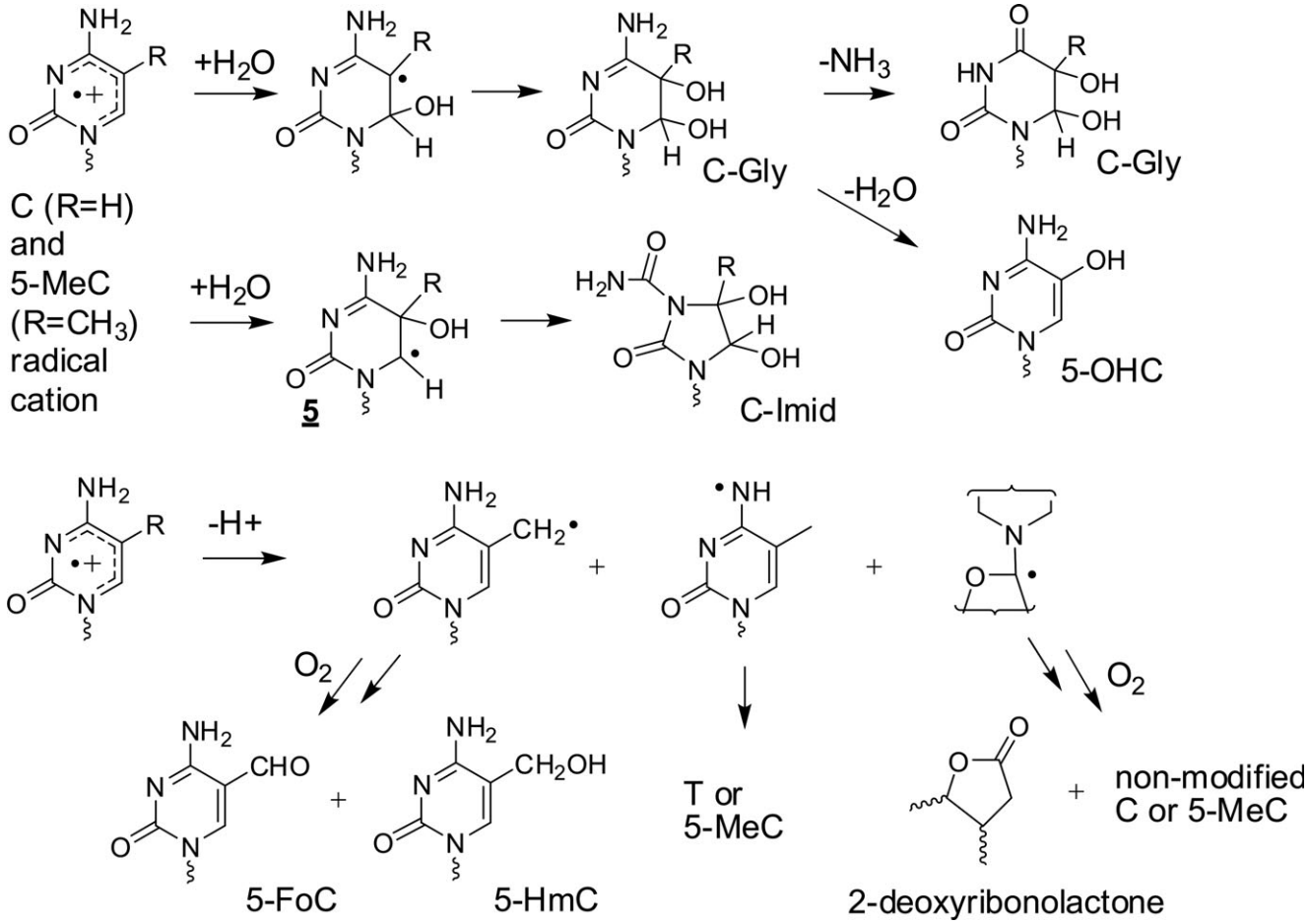


**Figure 3.** Main decomposition pathways of the cytosine and 5-methylcytosine radical cations through hydration and deprotonation in aerated aqueous solutions.

(*e.g.* in the presence of reductants and reducing metal ions) to generate the 2′-deoxyribonucleoside derivative of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapydGuo). Because 8-oxodGuo itself has a relatively low oxidation potential, it is highly susceptible to further one-electron oxidation, which leads to the formation of spiroiminodihydantoin derivatives through an acyl shift rearrangement of transiently generated 5-hydroxyl adducts (18,58). Similar to the hydration reaction, the hydroxymethyl group of nucleosides (59) and numerous amino-containing nucleophiles efficiently add to the electrophilic C8 position of $dGuo^{\cdot+}$ (60). In contrast to hydration, deprotonation gives rise to a strongly oxidizing G(-H)$^{\cdot}$ intermediate, which decays into two main and well-characterized purine fragmentation products: 2,2-diamino-4-[(2-deoxy-*β*-D-*erythro*-pentofuranosyl)amino]-5(2*H*)-oxazolone and its precursor, 2-amino-5-[(2-deoxy-*β*-D-*erythro*-pentofuranosyl) amino]-4*H*-imidazol-4-one (9,18). The mechanism of formation of the latter products involves initial addition of $O_2^{\cdot-}$ to a likely C5 carbon-centered radical followed by a series of subsequent rearrangements (61). It should be noted that the formation of 8-oxodGuo is a minor reaction in the riboflavin-mediated photosensitized ionization of dGuo in aqueous solutions (62) but gains paramount importance in double-stranded DNA (63) due to base stacking and base pairing (10,36).

*2′-Deoxyadenosine (dAdo).* One-electron oxidation of dAdo leads to the corresponding dAdo radical cation ($dAdo^{\cdot+}$), which undergoes hydration into a redox-ambiguous 8-hydroxy-7,8-dihydroadenyl radical and deprotonation into a strongly oxidizing dAdo(-H)$^{\bullet}$. The hydration reaction involves analogous pathways of decomposition and final products as those observed for dGuo radical cations (Fig. 4), that is 8-oxodAdo and FapydAdo (64,65). Indeed, the latter products appear to represent the majority of stable lesions arising from one-electron oxidation reactions. In the case of FapydAdo, opening of the imidazole ring at C8-N9, and the lack of substitution at N1, permits epimerization and sugar rearrangement of the initial beta-2-deoxyribose structure into three other nucleosides with corresponding alpha furanose and alpha/beta pyranose configurations. In contrast to $dGuo^{\cdot+}$, the deprotonation reaction of $dAdo^{\cdot+}$ involves loss of the proton at N4 resulting in a highly oxidizing N6-aminyl radical. The latter radical was independently generated in aqueous solution by near-UV photolysis of $N^6$-phenylsulfenyl and $N^6$-phenylhydrazone derivatives of dAdo in aqueous solution (66). Therefore, N6 aminyl radicals were observed to undergo mainly H-atom abstraction back to dAdo and deamination to give 2′-deoxyinosine, the N6-deaminated derivative of dAdo. In a molar excess of dAdo, however, there was evidence for the formation of novel dAdo-dAdo dimeric products likely connected though N3 or N6 positions (66). Recently, near-UV photolysis of an N6-substituted alkoxylamine adenine derivative was shown to yield the corresponding N6-aminyl radical by Norrish type I cleavage (44). An interesting

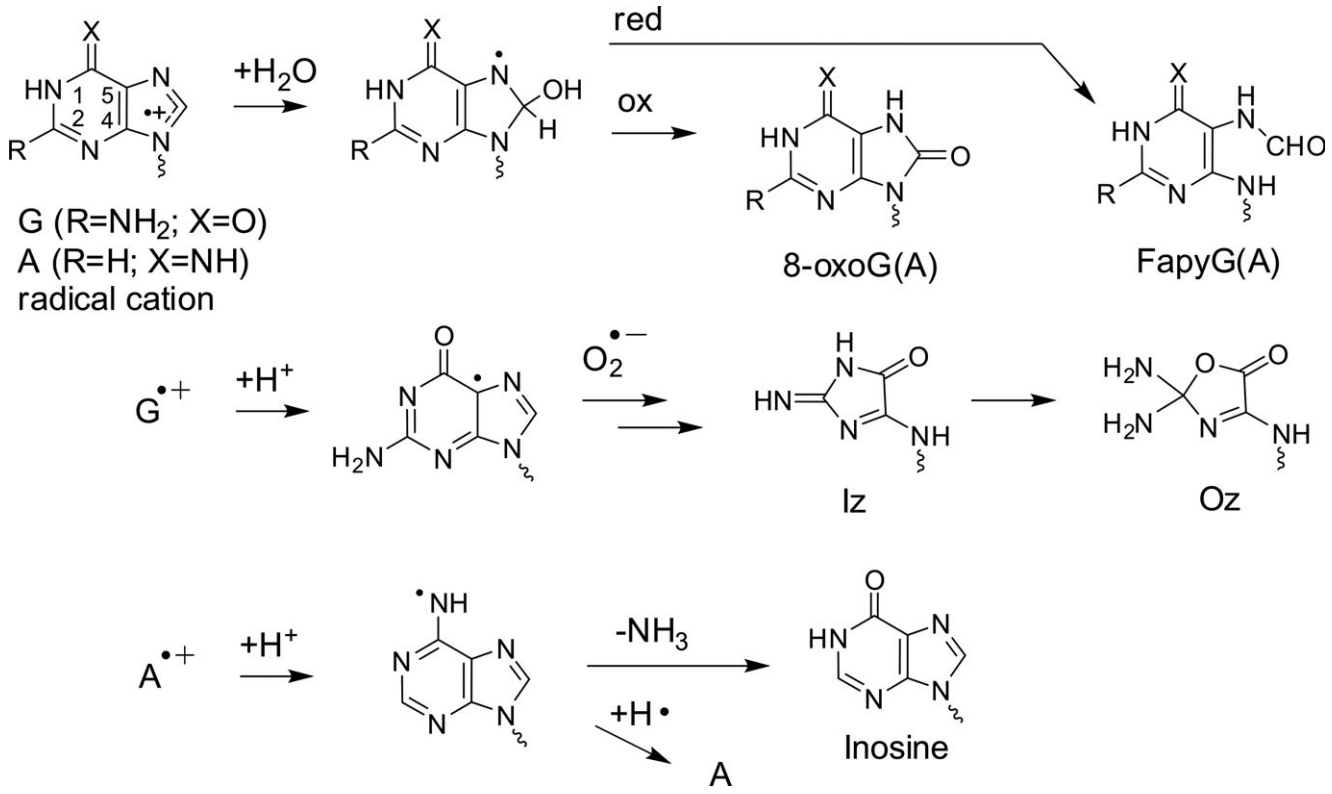


**Figure 4.** Main reactions of the guanine and adenine radical cations in aerated aqueous solutions following initial hydration or deprotonation.

aspect of this reaction is the lack of formation of secondary radicals. Using this reaction, Greenberg and coworkers reported the formation of tandem lesions and electron transfer phenomenon depending on the flanking nucleobase in duplex oligonucleotides (47,67).

## MAIN REACTIONS OF BASE RADICAL CATIONS IN ISOLATED DNA

### Oxidatively generated lesions

The first detailed mechanistic study on the chemical reactions of the guanine radical cation ($G^{\cdot+}$) in isolated DNA involved visible light–excited riboflavin, a well-documented type I photosensitizer and one-electron oxidant (63). Thus, hydration of $G^{\cdot+}$ was found to give rise to [$^{18}$O]-8-oxodG when the photosensitization was performed in [$^{18}$O]-$H_2O$. Monophotonic ionization of nucleobases upon exposure of plasmid DNA to 193 nm laser pulses in aqueous solutions predominantly generated guanine oxidation products, as revealed by the detection of formamidopyrimidine-DNA glycosylase (Fpg)-sensitive sites that are formed in a greater yield than oligonucleotide strand breakage (68,69). The formation of 8-oxodGuo has also been reported in duplex DNA following 248 nm excimer laser irradiation in frozen aqueous solutions. This was rationalized in terms of biphotonic ionization of guanine giving rise to $G^{\cdot+}$, the precursor of 8-oxo-7,8-dihydroguanine (8-oxoG) but not that of DNA strand breaks (70). Several subsequent studies have implicated high-intensity 266 nm ns and picosecond (ps) laser photolysis to induce two-quantum ionization of purine and pyrimidine nucleobases in isolated DNA that has led to the formation of oxidatively generated base damage (34,35,37).

It is worth noting that the term two-quantum (biphotonic) excitation used here is a synonym of consecutive two-step absorption through a real intermediate step (resonant two-photon excitation). The meaning is different from the term two-photon (biphotonic) absorption used in nonlinear optics designating absorption through a virtual intermediary state as used for example in biphoton confocal microscopy (71). Taking into account the lifetimes of the excited states of nucleobases (<1 ps for singlet state $S_1$, ~1 μs for triplet state $T_1$) and the very low value of the intersystem crossing yield ~1.5% for purine bases and fivefold to 10-fold lower for pyrimidine bases (72), the intermediate state can be either $S_1$ in case of picosecond and femtosecond laser pulse duration or $T_3$ in case of a ns laser pulse duration (26,28,72). In all cases, the energy of two 266 nm laser quanta exceeds the ionization potential of free bases (DNA) in solution and is sufficient to ionize all of them (28). It should be noted that $S_1$ photoexcited pyrimidine bases decay presumably via two distinct channels. The first one deals with direct internal conversion of the $^1\pi\pi^*$ state to $S_0$ with $\tau$~1 ps, while the second channel, which accounts for 10–50% of all de-excitation events, is assigned to the lowest-energy $^1n\pi^*$ state decay with a lifetime of 10–150 ps (73). This can explain the higher biphotonic photodamage yield of monomeric pyrimidines with respect to purines upon 30 ps laser photolysis (28,34,72).

*Identification of single oxidation base products.* As observed for isolated 2′-deoxyribonucleosides, an increase in the intensity of 266 nm ns pulses within the energy range 20–350 mJ cm$^{-2}$ leads to an enhanced formation of 8-oxodGuo and other biphotonic oxidatively generated purine and pyrimidines nucleosides at the expense of monophotonically induced bipyrimidine photoproducts (35,37,74). A plateau in the quantum yield of overall

oxidized nucleosides ($\phi = 0.19\%$) and bipyrimidine photoproducts ($\phi = 0.11\%$) was almost reached at an intensity of 350 mJ $cm^{-2}$ (37). As expected, the intensity dependence for the former biphotonic lesions displayed a sigmoidal shape in contrast to the mirror symmetrical decreasing shape for monophotonic bipyrimidine photoproducts. The majority of the bipyrimidine photoproducts are formed in the long-lived triplet state $T_1$ with a time constant no shorter than the laser pulse duration of 5 ns. Evidence was provided from HPLC–electrochemical detection, and the measurement of Fpg-sensitive sites that 8-oxodGuo was the predominant base lesion from either ns or ps laser irradiation of calf thymus DNA and a 37-mer duplex oligonucleotide (34). Application of an accurate and versatile HPLC-ESI-MS/MS method (75) has allowed the measurement of several oxidized purine and pyrimidine 2′-deoxyribonucleosides in isolated DNA upon UVC ns laser irradiation in aerated aqueous solutions (35,37). In addition to overwhelming 8-oxodGuo, the products consist of 8-oxo-7,8-dihydro-2′-deoxyadenosine (8-oxodAdo) and three thymidine oxidation products including 5,6-dihydroxy-5,6-dihydrothymidine (ThdGly), 5-(hydroxymethyl)-2′-deoxyuridine (5-HmdUrd) and 5-formyl-2′-deoxyuridine (5-FodUrd). The formation of the five oxidized 2′-deoxyribonucleosides that were generated at two different laser pulse intensities was found to be linear with the applied dose, and the quantum yields of formation are reported in Fig. 5 (37). In both cases, 8-oxodGuo is the main oxidation product. The DNA conformation is another major parameter that modulates the distribution of nucleoside oxidation products. The ratio 8-oxodGuo/other oxidized nucleosides was found to decrease in partially heat-denatured duplex DNA while an opposite trend was observed when polyamines were added as DNA compacting agents to the aqueous solutions (37).

The mechanism of formation of oxidized nucleobases in isolated DNA is likely to be similar to that already discussed in the section devoted to one-electron oxidation reactions of free 2′-deoxyribonucleosides. Interestingly, dedicated information has been gained on the properties and reactivity of $G^{\cdot+}$ in double-stranded DNA from both theoretical and experimental studies. The pseudo–first-order rate of $G^{\cdot+}$ hydration has been proposed to take place according to a counterion-assisted proton shuttle pathway (76) with a rate constant of $6 \times 10^4$ $s^{-1}$ (77). The nucleophilic addition of $H_2O/OH^-$ at C8 of the guanine moiety leads to transient 8-hydroxy-7,8-dihydroguanyl radical that upon one-electron oxidation and deprotonation generates 8-oxodGuo. Competitively, one-electron reduction of the latter guanyl radical, a minor reaction in aerated aqueous solution, gives rise to 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG). As a striking quantitative difference, the quantum yield of 8-oxodGuo in duplex DNA is about two orders of magnitude more elevated than that observed for free dGuo. There are three main reasons to explain the higher efficiency of 8-oxodGuo formation in a more organized structure such as duplex DNA. Stabilization of the DNA double-stranded structure is known to enhance the formation of 8-oxodGuo at the expense of the other oxidized nucleosides as the result of a charge transfer process that favors the redistribution of the positive holes on the guanine moieties. It has also been shown that the dynamic equilibrium between $G^{\cdot+}$ and the highly oxidizing $G(-H)^{\cdot}$ radical, its deprotonated form, is shifted to the radical cation precursor in native DNA (78). This is in agreement with the eight-fold enhancement of 8-oxodGuo formation in duplex DNA with respect to single-stranded DNA (78). This is accompanied by the formation in single-stranded oligonucleotides and free dGuo of 2,2-diamino-4-[(2-deoxy-*β*-D-*erythro*-pentofuranosyl)amino]-5(2*H*)-oxazolone (dOz) through a complex multistep mechanism (9,11) that is initiated by the addition of $O_2^{\cdot-}$ to $G(-H)^{\cdot}$ (79). The latter radical that is predominantly generated by one-electron oxidation of free dGuo has

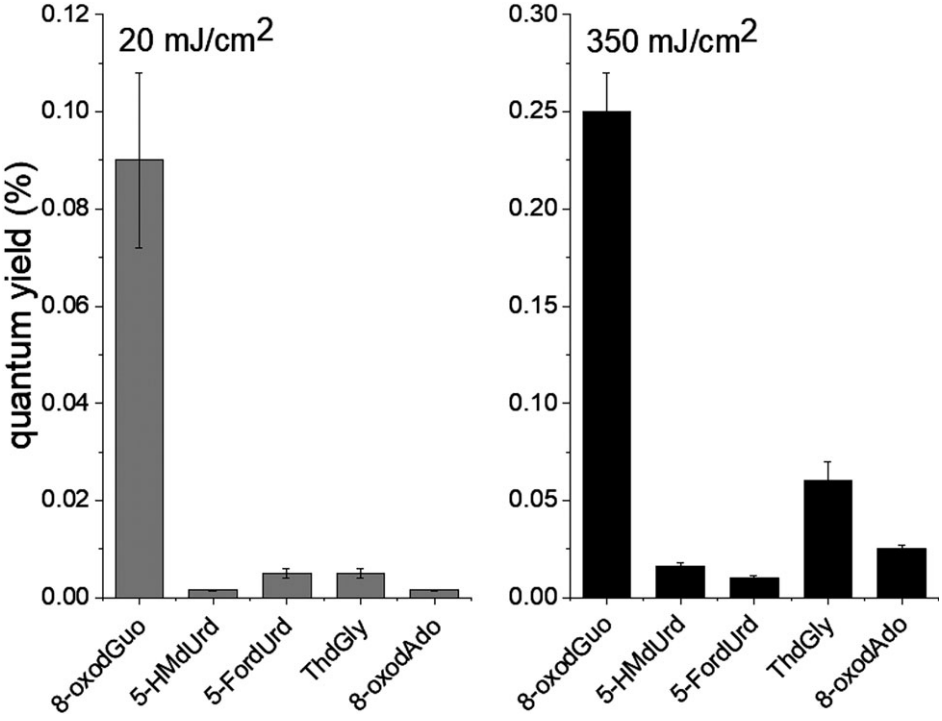


**Figure 5.** Distribution of the main oxidized 2′-deoxyribonucleosides formed in isolated DNA upon exposure to ns UV laser pulses of two different intensities (20 and 350 mJ $cm^{-2}$ respectively) in aerated aqueous solutions (37).

been shown to be an efficient oxidant of 8-oxodGuo (62). This leads to the rapid degradation of 8-oxodGuo as soon it is generated, a reaction that however is not efficient in DNA since the reactants are separated by too far a distance in most cases.

*Hole transfer and sequence effects.* Denaturing gel electrophoresis, HPLC-EC analysis and laser flash photolysis (34,78,80) provided strong support for the significant implication of base pairing and stacking in the spatial factors governing $G^{+\cdot}$ chemistry through competitive pathways involving initial deprotonation and hydration leading to guanine oxidation products. As a general observation, deprotonation of the guanine radical cation is predominant in single-stranded DNA (78) and base contexts including CGC, CGT, identified as non-B sequences, and proximal flexible steps ATAT. An enhancement of the deprotonation pathway was also observed upon local DNA structural destabilization induced by the presence of base mismatches. In contrast, competitive hydration and formation of 8-oxodGuo are favored in well-stacked regions, including repeated G runs. The factor responsible for the observed selective biphotonic $G^{+\cdot}$ generation and its heterogeneous distribution under ns laser photolysis of DNA has been tentatively attributed to the occurrence of $S_1$ and/or $T_1$ excited state energy transfer followed by hole migration and subsequent trapping by guanine bases (74). It is noteworthy that these nonradiative phenomena are strongly sequence- and structure-dependent. Indeed, the occurrence of excited state exciton formation and nonradiative energy migration (81–92) as well as long-range hole migration with localization at guanine residues (91–98) was suggested to explain the heterogeneity of excitation/charge distribution within DNA; however, several aspects still remain matters of debate and controversy. The sequence/secondary structure-dependent heterogeneity of $G^{+\cdot}$ distribution upon laser biphotonic photolysis of DNA was rationalized on the basis of kinetic equations analysis, bringing into play electronic excitation nonradiative energy transfer-mediated biphotonic excitation and hole migration toward guanines (74). Briefly, the following working model for nanosecond photolysis of DNA has been proposed (74). In the initial step, an arbitrary base A, T, C or G is excited by one-photon absorption to $S_1$ that subsequently is converted to $T_1$ by intersystem crossing. During the laser pulse, the excitation energy migrates by either S-S or T-T nonradiative transfer, trapped by a neighboring base that becomes $T_1$-excited and is further photo-ionized with subsequent electron ejection into the bulk. Following hole migration, presumably by a combination of single-step superexchange and successive electron hopping (77,92,95), the radical cation is trapped by a neighboring guanine, exhibiting the lowest ionization potential. The $G^{+\cdot}$ thus formed undergoes competitive hydration or deprotonation giving rise to the respective stable modifications. Note that thymine, having the lowest $T_1$, is the most likely energy trap precursor of the primary radical cation. The latter is compatible with the observed laser-intensity–dependent decrease in cyclobutane thymine dimers (36,74).

*Footprinting applications.* High-intensity 260 nm laser pulses induce DNA sequence- and structure-dependent modifications (35,74,80) through both mono- and biphotonic excitation of nucleobases. Footprinting methods are based on the detection of oxidized bases by induced DNA cleavage at the site of the lesions by either alkali or enzymatic treatment. Mapping of both biphotonic oxidatively generated lesions and monophotonic pyrimidine lesions resulting from exposure to a single UV laser pulse enables visualizing, at the nucleotide level, the local DNA structural "signature" of alterations in DNA resulting from temperature-induced local melting (99) or specific interactions with proteins, that is UV laser footprinting (100–102). The advantages of using a single UV laser pulse instead of conventional UV lamp radiation for photofootprinting experiments include the following:

1 the ability to perform time-resolved studies by synchronizing the laser pulse with either a stopped flow or a $T^0$ jump system.
2 the formation of a broader spectrum of DNA damage including a combination of mono- and biphotonic lesions.
3 a high sensitivity to DNA conformational changes.

Monitoring the temperature dependence of UV laser-induced hot piperidine (mostly Oz lesions) and Fpg-sensitive lesions (8-oxoG and FapyG) provided a unique tool to probe local helix-coil melting profiles at individual sites within short DNA fragments. This was found to reflect local small-scale breathing motions and unstacking single dinucleotide steps prior to base pair opening (99). This approach, combined with the laser-induced DNA–DNA interstrand cross-link formation at guanines, was successfully applied to address the bubble nucleation and structural effects induced by thermal fluctuations in short DNA fragments (103,104). Evidence was provided that the effect of structural perturbations exhibited by AT-rich low stability regions, present in certain common transcription initiation regions, influences the properties of DNA within a distant range of at least 10 bp (103).

The laser photofootprinting technique has been applied to study the binding of transcription factors to DNA. Using a combination of DNase1 and UV laser footprinting, it was shown that the high-mobility group box transcription factor HBP1, expressed in a differentiation-dependent manner, was involved in the activation of H10 gene expression by specific interactions with the highly conserved GGTCC H1.0 H4 box. This suggests that HBP1 would mediate a link between the cell cycle control machinery and cell differentiation signals (105). NF-$\kappa$B homodimer and heterodimer binding specificity and efficiency to multiple DNA target sequences were determined by UV laser footprinting. The results enabled validation of the large-scale EMSA-seq data (106). Biphotonic UV laser footprinting has been applied to study the interaction of NF-$\kappa$B p50 homodimers with its consensus sequence within the context of the nucleosome (107). Interestingly, it was shown that NF-$\kappa$B p50 homodimer is able to bind to its recognition sequence, when it is localized at the edge of the core particle, but not when the recognition sequence is positioned at the interior of the nucleosome. Remodeling of the nucleosome by the "Remodel the Structure of Chromatin" (RSC) complex was not sufficient to allow binding of NF-$\kappa$B to its recognition sequence located in the vicinity of the nucleosome dyad. However, RSC-induced histone octamer sliding allowed clearly detectable binding of NF-$\kappa$B with the shifted particle. It was also found that NF-$\kappa$B was able to displace histone H1 and prevent binding to the nucleosome (108). Noteworthy is that specific binding of other transcription factors, namely PU.1 and Sox6, to their DNA recognition sequences has been investigated by UV laser biphotonic probing. Interestingly, the latter factor was shown to invade the core nucleosomal DNA

independently on the translational position of the target sequence with a similar efficiency as naked DNA with the exception at the nucleosome dyad axis (results to be published). This result clearly defines Sox6 as a pioneering transcription factor without the need of nucleosome remodeling for target sequence recognition (109).

### Intrastrand and interstrand cross-links

Other nucleophilic addition reactions of the guanine radical cation besides hydration have been identified in DNA upon exposure to one-oxidant agents including $CO_3^{\cdot -}$, bi–photo-ionized 8-aminopurine and high-intensity 266 nm intensity UV laser pulses. DNA intrastrand cross-links (ICLs) between guanine and either adjacent thymine (Fig. 6) or a distant thymine, separated by a single cytosine, have been found to be generated in short oligonucleotides and isolated DNA upon one-electron oxidation of guanine. The formation of the resulting G*T* and G*CT* intrastrand adducts that were unambiguously characterized on the basis of extensive NMR and MS measurements have been rationalized in terms of nucleophilic attachment of thymine through its N3 position to $G^{\cdot +}$ at C8 (110,111). Information was recently gained from model studies on the possibility that two guanine–thymine lesions are removed by two different repair proteins. The bulkier G*CT* cross-link was excised four-fold more efficiently than the G*T* ICL by nucleotide excision repair (NER) enzymes of human HeLa cell extracts (112). It was also shown that both G*CT* and G*T* lesions can be removed from 17-mer DNA duplexes in HeLa cell extracts by base excision repair pathways (113).

Evidence for ns UVC laser-induced DNA interstrand cross-links (ICLs) at CG pairs of gene promoter regions was provided by sequencing polyacrylamide gel electrophoresis analysis showing characteristic slowly migrating bands (103). It is likely that the biphotonic formation of ICLs, whose unambiguous characterization remains to be established, would arise from the nucleophilic addition of the exocyclic C4 amino group of cytosine to C8 of initially generated $G^{\cdot +}$ positioned on opposite strands (114).

### DNA–protein cross-links

Exposure of DNA–protein complexes to UVC ns and ps laser irradiation in aerated aqueous solutions has been shown to induce with a high efficiency the formation of DNA–protein cross-links (DPCs) that received numerous biochemical applications as further discussed below.

*Mechanism of formation.* DPCs represent another relevant class of oxidatively generated DNA damage. The expected major biological relevance of DPCs, particularly in radiation biology, has been for a long time a matter of debate concerning the putative mechanisms involved in their formation (115,116). However, the postulated recombination reaction between vicinal base and amino acid radicals whose efficiency would be strongly affected by the presence of oxygen has not received experimental support due to the lack of unambiguous characterization of any radiation-induced DPCs. The situation has been at least partly clarified with the isolation and structural assignment of a guanine–lysine

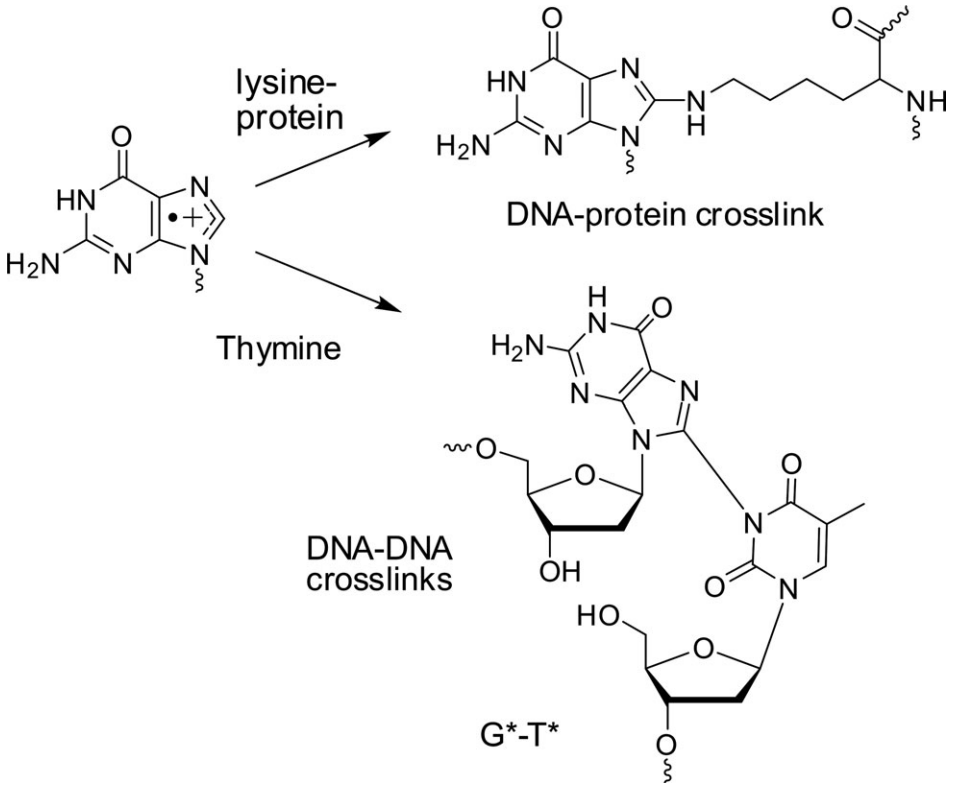


**Figure 6.** Complex modifications arising from nucleophilic additions to guanine radical cation: G*T* intrastrand, interstrand cross-links and DNA–protein cross-links (the G*-T* cross-link was detected after enzymatic digestion of DNA and likely contains an undamaged nucleotide bridging the cross-link in DNA).

adduct (su. 6) arising upon riboflavin-mediated photosensitization of TGT trinucleotide in the presence of KKK trilysine peptide (59). The mechanism for this efficient photoreaction that was recently extended to polyamines (117) was rationalized in terms of nucleophilic addition of the ε-amino group of central lysine to C8 of $G^{\cdot+}$ followed by one-electron oxidation. It may be noted that under these experimental conditions, competitive hydration giving rise to 8-oxoG was at best a minor process. This appears to be a relevant alternative to the radical recombination mechanism that remains however to be challenged in cellular DNA in the presence of one-electron oxidants including $CO_3^{\cdot-}$, potassium bromate or the direct effect of ionizing radiations (10,18).

*Biochemical applications.* The increasing interest in DPCs is dictated by their use as relevant markers in the studies of DNA (RNA)–protein interactions both *in vitro* and in the cellular environment (118). UV laser cross-linking exhibits several advantages over techniques using low-intensity light sources determined by the high amount of photons delivered by the laser in a single-nanosecond or single-picosecond pulse. This allows the protein–DNA cross-linking reaction to operate via a biphotonic mechanism through the intermediary of highly reactive radical cations of nucleobases leading to the formation of DPCs than low-intensity UV irradiation (30). The cross-linking reaction itself is completed in much <1 μs, which avoids the possibility of artefactual cross-linking of UV-damaged molecules and permits the trapping of rapid dynamic changes in protein–DNA interactions (119). A strong influence of pulse intensity and pulse length on the cross-link yield has been observed. As a striking observation, the highest efficiency of progesterone receptor cross-linking to DNA was achieved with femtosecond pulses (120). In contrast, the cross-linking yield of TATA-binding protein, glucocorticoid receptor and heat shock factor to free oligonucleotides was found to be independent on the laser pulse duration (121).

UV laser cross-linking has been used *in vitro* for studying different aspects of protein–DNA interactions including measurement of binding constants, determination of protein–DNA contact points, assessment of the size of the protein–nucleic acid complexation site (119,122). The UV laser cross-linking method has permitted immobilizing the transient DNA-ATPase complex in the T4 DNA replication system, hence permitting one to investigate the dynamics of protein–DNA interactions (119) The technique was also successfully applied in kinetic studies for the interaction of *E. coli* RNA polymerase with the promoter DNA sequence (123) as well as for the interaction of TATA-box binding protein (TBP) assembly with the adenovirus E4 promoter (124). The technique provides unique information that is not accessible using conventional biochemical approaches. Thus, histone–DNA cross-linking induced by UV laser irradiation of nucleosomes was found to occur only via the nonstructural histone NH tails that interacts with linker DNA (125,126). This finding has allowed one to demonstrate that the tails are not released from nucleosomal DNA upon histone acetylation, a question that has been debated for many years in the literature (127). Interestingly, binding of individual transcription activators Sp1, NF-kB1, LEF-1 and USF as well as SWI/SNF remodeling of the HIV-1 nucleosome does not induce the stable release of histone NH2 tails from nucleosomal DNA (128). A nanosecond UV laser cross-linking assay has been used to study in real time the dynamics of progression of the SWI/SNF and the glucocorticoid receptor (GR) binding at the MMTV promoter assembled in an array of correctly positioned nucleosomes (129). A preparative procedure for single-pulse UV laser cross-linking of chromosomal and regulatory proteins to DNA within whole cells has also been developed (130). This procedure, in combination with immunochemical techniques (laser X-ChIP-qPCR), was used to detect the presence of specific proteins on DNA sequences of interest in whole cells. These included RNA pol II and the androgen receptor (AR) (131), as well as RNA pol V and AGO4 in nuclei isolated from plant cells (132). The above examples demonstrate the utility of the UV laser cross-linking for gaining relevant structural and conformational information that cannot be obtained using either the conventional UV irradiation or formaldehyde cross-linking techniques. It is worth noting that biphotonic cross-linking occurs not only in DNA–protein complexes but in RNA–protein complexes as well (133,134).

## BIPHOTONIC IONIZATION OF CELLULAR DNA

Human cells have been exposed to high-intensity nanosecond 266 nm pulses in order to gain mechanistic insights into the formation of oxidized bases upon ionization of the DNA pyrimidine and purine components (25,135). The oxidized 2′-deoxyribonucleosides were measured by the accurate isotopic dilution HPLC-ESI-MS/MS method operating in the multiple reaction monitoring mode under suitable conditions of DNA enzymatic digestion and prevention of spurious artefactual oxidation (18,136,137).

### Predominance of 8-oxo-7,8-dihydroguanine

In an earlier study, it was found that exposure of THP1 human monocytes to high-intensity nanosecond UVC laser pulses gave rise to the predominant formation of 8-oxodGuo together with much smaller amounts of thymidine oxidation products including ThdGly, 5-HmdUrd and 5-FoUrd (25). The respective yields that are expressed in frequency of lesions per $10^9$ nucleosides per mJ are the following: 8-oxodGuo (1.29) > ThdGly (0.17) > 5-HmdUrd (0.06) > 5-FodUrd (0.02). A similar distribution of oxidized 2′-deoxyribonucleosides that also includes 5-OHdCyd one of the main 2′-deoxycytidine radical oxidation products was measured in the DNA of human HeLa cells upon nanosecond 266 nm laser pulse irradiation at intensities of 50 and 280 mJ $cm^{-2}$ pulse, respectively (Table 1) (135). The increase in laser intensities is accompanied by a concomitant increase in the yields of oxidized

**Table 1.** DNA modification frequency of oxidized 2′-deoxyribonucleosides* measured by HPLC-ESI-MS/MS in the DNA of HeLa cells upon exposure to intense ns 266 laser irradiation†.

| | Laser intensity (mJ $cm^{-2}$ $pulse^{-1}$) | | |
|---|---|---|---|
| DNA lesions | 50 | 280 | Nonirradiated controls |
| 8-oxodGuo | 263 ± 85 | 936 ± 28 | 4.8 ± 3.4 |
| 5-FodUrd | 28.4 ± 3.8 | 71.3 ± 5.1 | 1.0 ± 2.0 |
| 5-HmdUrd | 28.4 ± 3.7 | 70.2 ± 4.8 | 1.9 ± 0.4 |
| 5-OH dCyd | 21.0 ± 7.2 | 51.0 ± 6.7 | 0.08 ± 0.19 |
| ThdGly | 11.9 ± 2.4 | 22.3 ± 2.8 | 1.9 ± 1.2 |
| G*-T* | 0.21 ± 0.11 | 1.19 ± 0.03 | 0.02 ± 0.05 |

*Expressed as the number of lesions per $10^9$ 2′-deoxyribonucleosides.
†Laser dose: 120 J.

nucleosides. This was rationalized in terms of a higher efficiency of biphotonic ionization of the bases as observed in isolated DNA (37). The overwhelming laser-induced 8-oxodGuo over Thd and dCyd oxidation products were already observed in native double-stranded DNA. This may be accounted for by charge migration of the positive hole within a few bases along the DNA chain with preferential trapping by guanine bases (91–98) that exhibit the lowest oxidation potential among canonical nucleobases (138,139). Interestingly, the above reported findings that constitute the two known examples of accurate measurements of one-electron base oxidation in cellular DNA provide strong indirect support for occurrence of efficient hole transfer in cellular DNA.

### GT cross-links

Formation of T*-G*, the tandem base lesion that was found to arise from nucleophilic addition of thymine through N3 to the guanine radical cation at C8 in isolated DNA, was detected in the HeLa cells after exposure to high-intensity UVC laser irradiation (135). This intramolecular reaction that occurs with a much lower efficiency than base oxidation (Table 1) constitutes another example of the wide panel of chemical pathways of $G^{\cdot+}$ already identified in both model compounds and isolated DNA (140).

## PERSPECTIVES

Comprehensive one-electron oxidation-mediated decomposition pathways of the five main DNA nucleobases have been inferred from detailed mechanistic studies either upon biphotonic ionization or type I photosensitization reactions. Extension of the studies to isolated DNA has allowed to demonstrate an enhanced formation of 8-oxodGuo at the expense of other one-electron purine and pyrimidine oxidation products. This is likely the result of redistribution of initially ionized sites through hole migration. Indirect confirmation of the occurrence of this charge transfer mechanism along oligonucleotide chains in cellular DNA upon high-intensity UVC laser irradiation was provided by the overwhelming formation of 8-oxodGuo. In addition, intrastrand cross-links between guanine and vicinal thymine have been characterized and measured in both isolated and cellular DNA. Other relevant nucleophilic reactions of $G^{\cdot+}$ that include DNA interstrand cross-links and DNA–protein cross-links have been shown to be operative in model studies. It remains however to demonstrate through the detection of suitable biomarkers that these later complex DNA lesions are generated as well in cellular DNA. This will await forthcoming studies.

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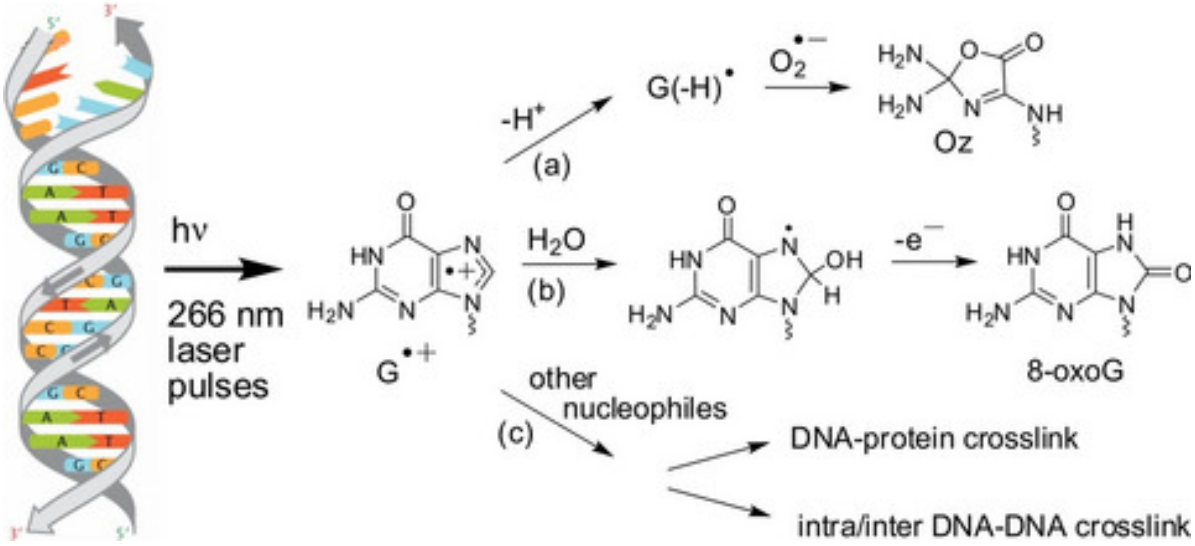


Main reactions of the guanine radical cation ( G · + ) in isolated and cellular DNA upon either direct biphotonic ionization or hole transfer from other purine and pyrimidine radical cations to guanine. (a) Deprotonation pathway. (b) Hydration pathway. (c) Other nucleophilic reactions.