High-Intensity UV Laser Photolysis of DNA and Purine 2′-Deoxyribonucleosides: Formation of 8-Oxopurine Damage and Oligonucleotide Strand Cleavage as Revealed by HPLC and Gel Electrophoresis Studies

D. Angelov,[†] A. Spassky,[‡] M. Berger,[§] and J. Cadet*[,§]

Contribution from the Institute of Solid State Physics, Bulgarian Academy of Sciences, 1784 Sofia, Bulgaria, Département de Recherche Fondamentale sur la Matière Condensée, SCIB/Laboratoire "Lésions des Acides Nucléiques", CEA/Grenoble, 17, rue des Martyrs, F-38054 Grenoble, Cedex 9, France, and Université René Descartes, UA 400, 45, rue des Saints Pères, F-75270 Paris, Cedex, France

Received May 27, 1997[⊗]

Abstract: Emphasis was placed in this work on the measurement of purine oxidation products generated upon nano- and picosecond UV laser biphotonic photolysis of 2′-deoxyadenosine, 2′-deoxyguanosine, calf thymus DNA, and a synthetic duplex oligonucleotide (37-mer) in aerated aqueous solutions. The overall formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine and 8-oxo-7,8-dihydro-2′-deoxyadenosine was determined using a HPLC-electrochemical detection assay. Denaturing gel electrophoresis analysis in association with a formamidopyrimidine−DNA glycosylase treatment was applied to reveal the sites recognized by this DNA repair enzyme. Both 8-oxo-7,8-dihydro-2′-deoxyguanosine and 8-oxo-7,8-dihydro-2′-deoxyadenosine were shown to be minor decomposition products of the related nucleoside purine radical cations in agreement with earlier observations. Interestingly, a dramatic increase in the yield of both photoproducts, this applying particularly to 8-oxo-7,8-dihydro-2′-deoxyguanosine, was observed in DNA. It should be noted that the yield of 8-oxo-7,8-dihydro-2′-deoxyguanosine was about 3-fold lower in heat-denatured DNA than in double-stranded DNA. These observations provide strong support to the significant involvement of base stacking and probably DNA solvatation in the chemical reactions of the purine radical cations. Other interesting information dealt with the similarity in the level of 8-oxo-7,8-dihydro-2′-deoxyguanosine and the number of formamidopyrimidine−DNA glycosylase sensitive guanine lesions. This strongly suggests that the latter formamidopyrimidine−DNA glycosylase purine nucleoside is the major DNA photodamaged product recognized by the DNA repair glycosylase. Another striking feature is the almost 10-fold decrease in the saturation dose E_s for the two-quantum ionization of the guanine base in double-stranded DNA as compared to that observed for free 2′-deoxyguanosine. This can be explained by either an enhancement of the quantum yield of photoionization from the intermediate excited state in DNA (φ_2) and/or hole migration with preferential trapping by guanine residues.

Introduction

It is now well established that exposure of DNA and related model compounds to either 248- or 266-nm high-intensity laser pulses gives rise to pyrimidine and purine radical cations as the result of biphotonic excitation.[1,2] It is also assumed that the latter reactive transient radicals are converted into stable oxidation products via two competitive pathways involving deprotonation and hydration.[3] Only limited information is available on the stable decomposition products generated by biphotonic laser photolysis of DNA components. One major exception deals with a preliminary report on the characterization of the main UV laser-induced photoproducts of thymidine (dThd) and 2′-deoxycytidine (dCyd).[4] It should be mentioned that detailed information on the chemical reactions of the pyrimidine radical cation of dThd, dCyd, and 5-methyl-2′-deoxycytidine was mostly inferred from type I photosensitization studies involving 2-methyl-1,4-naphthoquinone as the photodynamic agent.[3−6] Significant progress in the determination of the major oxidation products deriving from the purine radical cations is also associated with photosensitization studies. Thus, it was found that riboflavin-mediated photosensitization of DNA leads to the significant formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo) through a type I mechanism.[7] Interestingly, 8-oxo-dGuo is not generated, at least in significant amounts, in free dGuo and short single-stranded oligonucleotides due to a fast deprotonation of the transient guanine radical cation.[8,9] The main oxidation product which arises from the latter pathway was characterized as an oxazolone derivative,[10,11]

* To whom the correspondence should be addressed. Telephone: (33)-4-76-88-49-87. FAX: (33)-4-76-88-50-90. E-mail: cadet@drfmc.ceng.cea.fr.
[†] Bulgarian Academy of Sciences.
[‡] Université René Descartes.
[§] CEA/Grenoble.
[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1997.

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S0002-7863(97)01728-9 CCC: $14.00

a highly alkali-labile compound.[12] It was shown on the basis of sequencing gel electrophoresis analysis that exposure of DNA to high-intensity laser pulses results in the formation of alkali-labile lesions[13,14] and DNA polymerase stops[15] at guanine sites. However, there is still a paucity of information on the chemical structure of the oxidative damage occurring upon photoionization of purine bases. One major exception deals with a recent study on the 248-nm excimer laser-mediated photoionization of oligonucleotides and free 2′-deoxyguanosine.[16] It was shown that 8-oxo-dGuo and unknown piperidine-labile guanine lesions are generated under these conditions. A more indirect evidence for the formation of 8-oxo-dGuo was provided by the measurement of formamidopyrimidine−DNA glycosylase (Fpg)-sensitive sites within 193-nm irradiated DNA fragments.[17] It was also reported that either mono- or biphotonic laser photolysis of DNA gives rise to strand cleavages. However, the process is at the best inefficient with respect to the formation of oxidative base damage and usual pyrimidine photoproducts including cyclobutadipyrimidines and pyrimidine (6-4) pyrimidone photoadducts.[1,16,18]

Emphasis was placed in the present work on the measurement of the quantum yield of photoinduced 8-oxo-dGuo and 8-oxo-dAdo in different purine substrates including 2′-deoxyguanosine (dGuo), 2′-deoxyadenosine (dAdo), calf thymus DNA, and a defined double-stranded oligonucleotide (37-mer). This was achieved using two different approaches including a HPLC-electrochemical detection assay and a denaturing gel electrophoresis method associated with a formamidopyrimidine−DNA glycosylase protein[19] treatment. The observed features are discussed in terms of efficiency of ionization of purine DNA samples and chemical reactivity of the related radical cations.

Experimental Section

Materials. 2′-Deoxyadenosine (dAdo), 2′-deoxyguanosine (dGuo), and calf thymus DNA were purchased from Sigma. Proteins were removed from calf thymus DNA by three successive phenol−chloroform extractions. 8-Oxo-dGuo and 8-oxo-dAdo were prepared according to slight modifications of reported procedures.[20,21] Oligodeoxyribonucleotides (37-mer) were prepared on an Applied Biosystem synthesizer and deprotected according to the manufacturer's procedure (for the sequence, see Figure 1). A final purification was achieved on a high-resolution 15% polyacrylamide/7 M urea denaturing gel. Oligonucleotides were 5′-end labeled using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Boehringer). The resulting radiolabeled oligonucleotide was purified by filtration on a Sephadex G-50 column and subsequently annealed with the corresponding complementary strand. Nonlabeled oligonucleotides, used for HPLC analysis, were annealed with the complementary DNA fragment in a 1:1 ratio. Controls were made on a nondenaturing 15% electrophoretic gel. The purified Fpg protein was a kind gift of Dr Serge Boiteux (CEA/Fontenay-aux-Roses, France). All other chemicals used were from the highest purity grade.

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9G:   5′-ATAAAAGTTACGCTAGGGATAACAGGGTAATATAACG-3′
4G:   3′-TATTTTCAATGCGATCCCTATTGTCCCATTATATTGC-5′
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Figure 1. Sequence of the 9G and 4G oligonucleotides.

Laser Apparatus. The fourth harmonic (λ = 266 nm) of two different types of Nd:YAG lasers was used as the source of UV radiation. The first one was a passively mode locked laser delivering single 30-ps pulses with an energy maximum of 8 mJ at 266 nm. The second far-UV source was a Q-switched laser providing 15-ns duration pulses with more than 30 mJ of energy maximum at 266 nm. The energy of the laser radiation was measured with a model R_j7100 (Laser Precision) calibrated pyroelectric energy meter (±5%) using the Fresnel reflections from a fused silica beam splitter positioned behind the sample. The beam fluence was varied by either using a focusing (f = 40 cm) quartz lens or changing the laser amplifier voltage together with detuning the harmonic generation crystals. The laser pulse dose was determined by using the measured energy and by assuming regular distribution of the energy across the laser beam. The diameter of the beam was determined by a photodiode array camera.

Laser Irradiation. Irradiation of dAdo, dGuo, and calf thymus DNA was carried out in 0.5-cm spectrophotometer fused silica cuvettes (optically thick solutions) under continuous stirring. The solutions of 2 mM purine nucleosides were prepared in bidistilled water (pH 6.5) whereas calf thymus DNA (1 mM concentration in nucleotides) was irradiated in 50 mM phosphate buffer (pH 7.6). For the different laser intensities, the total irradiation energy was selected in the linear range of the quantum yield response. The 37 bp DNA fragments, dissolved in TE buffer (10 mM Tris (pH 7.6), 1 mM EDTA, 40 mM NaCl), were irradiated as 10-μL aliquots in small 0.65-mL siliconized Eppendorf tubes (optically thin layer). The laser beam diameter was reduced to 0.25 cm (S = 0.05 cm^2) using a circular diaphragm to match with the area of the irradiated sample. [^{32}P]-5′-Labeled DNA (0.02 pmol) was irradiated with one pulse at the maximum fluence of 0.1 J·cm^{-2} (I = 6.6 MW·cm^{-2}). In parallel the number of pulses at lower intensities was increased to a total irradiation dose producing similar DNA degradation (below 10−15%) for all intensities used. For HPLC analysis, 1.83 μg of nonlabeled 37 bp DNA was dissolved in 50 μL of TE buffer and aliquots of 10 μL were transferred prior to irradiation into five Eppendorf tubes to ensure an optically thin irradiation layer—D = 0.133 in the 0.2-mm irradiation path length (assuming ϵ_{DNA} = 6000 M^{-1} cm^{-1} at 266 nm). All five samples were exposed to two pulses having energies of 5.6 mJ each (I = 7.5 MW·cm^{-2}). After irradiation, the aliquots were transferred in one tube and lyophilized prior to HPLC analysis.

HPLC-EC Determination of 8-Oxo-dGuo and 8-Oxo-dAdo in Photoirradiated Aqueous Solutions of Purine 2′-Deoxyribonucleosides. After laser irradiation, the solutions of dAdo and dGuo were evaporated to dryness. The resulting residues were dissolved in about 200 μL of water prior to HPLC analysis for their content in 8-oxo-dAdo and 8-oxo-dGuo. The HPLC system which was used for this purpose consisted of a LKB model 2150 pump (Pharmacia) equipped with a Rheodyne loop injector, Model 7125. The electrochemical detection was achieved using a dual LC-4B/17 AT amperometer detector (Bioanalytical System). The separation of 8-oxo-dAdo and 8-oxo-dGuo was carried out on an analytical octadecylsilyl silica gel column (4.6 mm i.d. × 250 mm, 5 μm) from Interchim. The eluent was a mixture of 0.05 M citrate buffer (pH 5) and methanol (80:20, v/v). The oxidation potential was set up at +850 mV for the detection

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of both 8-oxo-dAdo and 8-oxo-dGuo.[22] However, the detection of 8-oxo-dGuo could be accomplished in a more specific way, using a lower value (650 mV) of oxidation potential.[23] The quantitative determination of unmodified dAdo and dGuo was achieved by UV measurement using a Merck Model L 4000 variable spectrometer set up at 254 nm. Quantification of normal and oxidized nucleosides was made using calibration curves.

Quantitative Measurement of 8-Oxo-dAdo and 8-Oxo-dGuo in Calf Thymus DNA and Duplex Oligodeoxyribonucleotide. Irradiated DNA samples were ethanol precipitated and redissolved in 100 μL of water for enzymatic digestion. This was achieved by adding to the latter solution 10 μL of 10 X (300 mM sodium acetate (pH 5.3), 10 mM zinc sulfate) and 5 μL of P1 nuclease ready to use (Boehringer). After incubation for 2 h at 37 °C, 12 μL of 10 X (2 M NH_4HCO_3, pH 9) and 2 μL of alkaline phosphatase (Boehringer) were added. The resulting hydrolysate was analyzed for their content in 8-oxo-dAdo, 8-oxo-dGuo, and normal nucleosides by applying the above-described HPLC assays.

Denaturing Gel Electrophoresis. The laser-irradiated [^{32}P]-5′-labeled 37 bp DNA fragments were incubated with 5 ng of Fpg protein for 45 min at 37 °C. Then, the solution was lyophilized and the resulting dry residue resuspended in formamide loading buffer. DNA fragments were heat denatured during 5 min at 95 °C and subsequently run on a 15% denaturing polyacrylamide mini gel. The same procedure, but without Fpg treatment, was applied for measuring frank DNA strand breaks. Following electrophoresis, gels were dried and autoradiographed by using a Phosphorimager apparatus (Molecular Dynamics). The overall radioactivity deposited in one hand and the radioactivity in the ladder sequence on the other hand were determined by volume integration of rectangles using the Image Quant software version 3.1 (Molecular Dynamics).

Determination of the Quantum Yields. The quantum yield (QY) for the formation of a product P may be defined in two ways.[24] Convention 1: $QY_{tot}(P)$ = number of products P per total number of photons absorbed. Convention 2: QY(P) = number of products P per number of photons absorbed only by the precursors of P. Total means photons absorbed by all molecules present in the solution. Here QYs were determined according to convention 2, with one exception that concerns the gel electrophoresis experiments, where QY_{tot} values are also reported. In the case of irradiation of optical thick samples (dAdo, dGuo, and calf thymus DNA), the total number of incident and absorbed photons are identical. To determine QY(8-oxo-dGuo) and QY(8-oxo-dAdo), the contents of dGuo (22%) and dAdo (28%) in calf thymus DNA were taken into account. In addition, a correction was made for the difference in molar extinction of dAdo and dGuo, assuming by analogy with free purine nucleosides, that $\epsilon_{dAdo} = 1.4\epsilon_{dGuo}$. In the case of irradiation of optically thin layers, the quantum yield QY is practically impossible to be directly determined. Therefore, we determined the quantum efficiency ($QE_{(tot)}$), which is the amount of photoproduct per (total) quanta absorbed under linear absorption approximation (for the definition, see refs 25 and 26). For the experiments dealing with the HPLC analysis of 37 bp DNA fragments, the total number of absorbed photons under linear absorption approximation was determined using the steady-state absorbance value, $D = 0.133$, in the 0.2-cm irradiation path length of the Eppendorf tube. Thus, the total number of photons absorbed by 1.83 μg of DNA was 2×10^{16}, whereas the number of photons absorbed by the guanine residues was 0.35×10^{16}. For the experiments involving [^{32}P]-labeled DNA and gel electrophoresis analysis, the quantum efficiency was calculated using the formula

$$QE_{tot} = R_L/(R_T L \sigma_1 E_{tot}) \quad (1)$$

where $\sigma_1 = 2.3 \times 10^{-17}$ cm^2 is the absorption cross section of DNA and guanine bases within DNA at 266 nm ($\epsilon = 6000$ M^{-1} cm^{-1}), E_{tot} is the total irradiation fluence expressed in photons·cm^{-2}, $L = 37$ is the number of bases in the 5′-labeled oligonucleotide, and R_T and R_L are the total radioactivity deposited and the radioactivity in the ladder sequence, respectively.

Results

Generation of 8-Oxo-dAdo and 8-Oxo-dGuo in Isolated dAdo, dGuo, and Calf Thymus DNA. The quantum yields for the generation of 8-oxo-dAdo and 8-oxo-dGuo versus the laser pulse intensity upon exposure of dAdo and dGuo to nano- and picosecond pulses are reported in Figure 2. The solid lines represent theoretical fits of the experimental points with the corresponding expressions[27] describing the biphotonic photolysis in an optical thick layer. Consideration of the slope of the curves which show an initial linear increase followed by a saturation unambiguously demonstrates the biphotonic character of the photoprocess involved. The values of the saturation fluences I_s are similar to those already reported for chromophore loss,[28] namely several tens of MW·cm^{-2} and several GW·cm^{-2} in the case of nanosecond and picosecond photolysis, respectively. The saturated (maximum) values of the quantum yields QY_{max} are very low and similar for both photoproducts. However, under the picosecond mode of irradiation, the quantum yields are about 5 times higher. Upon addition of an •OH radical scavenger (5% MeOH), significant (3−5-fold) decreases in the yields of both 8-oxo-dAdo and 8-oxo-dGuo are observed under both irradiation conditions. It is likely that the •OH radicals which are involved in the formation of 8-oxo-dAdo and 8-oxo-dGuo arise from water photosensitization.[1,29] Interestingly, several major changes were noted in the photochemistry of naked DNA (Figure 3) by comparison with isolated nucleosides (Figure 2) (see also Table 1). The quantum yields QY_{max} for 8-oxo-dGuo in DNA increase about 50 times (without MeOH) and 200 times (with MeOH) compared to those observed for free nucleosides. The quantum yield for the formation of 8-oxo-dAdo in DNA is about 2−3 times lower than that for 8-oxo-dGuo. Interestingly, within the experimental error range, the presence of •OH radical scavengers (either 2−5% MeOH or *t*BuOH) has almost no influence on the formation of both 8-oxo-dAdo and 8-oxo-dGuo in DNA. The saturation fluence of the quantum yield, which is defined as the fluence corresponding to $0.63QY_{max}$, for 8-oxo-dGuo in DNA is almost 1 order of magnitude lower than in free dGuo. In addition, QY(8-oxo-dGuo) was found to be about 2.5 times lower in single-stranded (heat-denatured) DNA than in double-stranded DNA (Table 1).

Generation of 8-Oxo-dGuo in 37 bp Duplex DNA. It was found that the irradiation of DNA (1.83 μg) with two pulses (I = 7.5 MW·cm^{-2}) leads to the formation of 5.7 ± 0.25 ng (or 1.2×10^{13} molecules) of 8-oxo-dGuo. Taking into account the number of photons absorbed (see the Experimental Section), the corresponding values for the quantum efficiency are QE_{tot}(8-oxo-dGuo) = 6×10^{-4} (total photon absorbed) and QE(8-oxo-dGuo) = 3.4×10^{-3} (photons absorbed by guanine residues only). Although the HPLC-electrochemical assay is very sensitive, a relatively large amount of purified DNA fragments (a few micrograms per analysis) is required for each determination. To obtain the intensity dependence of the quantum yield, a more sensitive assay was used. This involved enzymatic incubation of irradiated DNA fragments with the DNA repair

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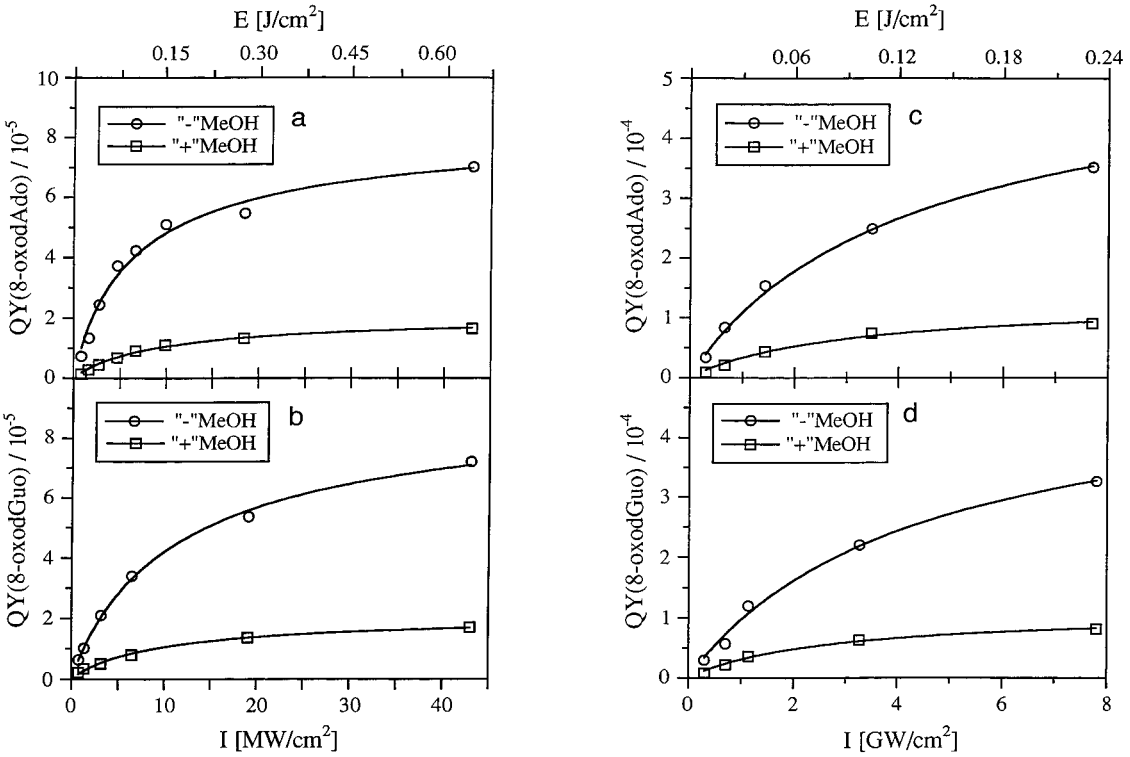

Figure 2. Laser pulse intensity (dose) dependence of the quantum yield QY on the generation of 8-oxo-dAdo (a, c) and 8-oxo-dGua (b, d) under nanosecond (a, b) and picosecond (c, d) laser photolysis of free dAdo and dGuo, respectively (optical thick layer, $c = 2$ mM). Circles: water. Squares: 5% methanol. Solid curves represent the best fits between the experimental data and eqs 16 and 22 taken from the ref 27.

Fpg protein followed by denaturing gel electrophoresis analysis (Figure 4). The latter assay takes advantage of the ability for the Fpg to recognize and quantitatively excise guanine lesions including 8-oxo-7,8-dihydroguanine and the related formamidopyrimidine derivative.[19,30] In addition, comparison of the present data with those obtained by HPLC analysis allowed the determination of the relative amount of 8-oxo-dGuo that is recognized by the Fpg protein with respect to other putative guanine lesions.

The 5′-labeled 9G oligonucleotide annealed with the complementary nonlabeled 4G strand was exposed to different laser fluences. High-resolution sequencing gel analysis revealed that only lesions located at guanine are cleaved by the Fpg repair enzyme.[31] The oxazolone compound, another major transformation product of the guanine radical cation,[3,10] is not recognized by the Fpg protein. However, the latter oxidative guanine lesion which is piperidine labile is likely to be generated upon UV laser photolysis of DNA.[31] It should be remembered that both Fpg-sensitive lesions and immediate strand breaks (sb's) are revealed simultaneously by denaturing gel analysis. Therefore, the irradiated samples were split into two aliquots; the first one was directly analyzed to establish QE_{tot}(sb), whereas the second aliquot was treated with the Fpg protein to determine Q_{tot}(Fpg+sb). Thus, the quantum efficiency of guanine lesions QE_{tot}(Fpg) recognized by the Fpg enzyme was obtained by subtracting QE_{tot}(sb) from QE_{tot}(Fpg+sb). The determination of QE(Fpg) (convention 2) implies taking into account the fact that there are eight guanine residues among the 37 bases in the ladder sequence. Consequently QE(Fpg) = QE_{tot}(Fpg) × 37/8.

The fluence−response curves of the quantum efficiencies are reported in Figure 5. It is obvious by considering the slopes of the curves that both immediate strand breaks together with Fpg-sensitive lesions originate from biphotonic excitation. The solid lines were obtained by least-squares approximation of the experimental points using eq 2 (see the Discussion and refs 27 and 28). It was found that QE(Fpg) = $3.5 \times 10^{-3} \pm 5\%$ for a fluence of 0.1 J·cm^{-2}.

The value of the quantum efficiency (QE(Fpg) = 2.4×10^{-3}) for the opposite 4G strand after exposure to one pulse ($E = 0.1$ J cm^{-2}) was determined in a similar manner. Taking into account these values, for the 9G/4G duplex, QE(Fpg) was found to be 3.2×10^{-3}. The maximum values of the quantum efficiencies extrapolated from the curves (QE_{max}) as well as the values at $E = 0.1$ J·cm^{-2} are reported in Table 1. It is important to note that QE(Fpg) and QE(8-oxo-dGuo), which were determined by electrophoresis and HPLC measurements, respectively, exhibit similar values within the experimental error (± 5%) for each determination (Table 1). Therefore, it may be concluded that the biphotonically generated guanine lesion recognized by the Fpg protein is 8-oxo-dGuo. Consequently, 8-oxo-dGuo is the only major Fpg-sensitive lesions of the guanine radical cation in double-stranded DNA.

It should be noted that no Fpg-sensitive sites were detected at any adenine residue,[31] although 8-oxo-dAdo is generated in a relatively high yield in duplex DNA upon exposure to UV laser irradiation. This is in agreement with previous results which showed that 8-oxo-7,8-dihydroadenine is not a substrate for the glycosylase activity of the Fpg protein.[30]

DNA Strand Breakage. The quantum efficiency of DNA strand breaks was determined by direct gel electrophoresis analysis (Figure 3). For the strand 9G it was established that QE(sb) = 4×10^{-4} ($E = 0.1$ J·cm^{-2}) and QE_{max}(sb) = 6.3×10^{-4} by extrapolation of the curve. For the opposite 4G strand, QE(sb) and QE_{max}(sb) were found to be 3.3×10^{-4} and 5.1×10^{-4}, respectively (data not shown). Thus, the averaged values for the 9G/4G duplex are QE(sb) = 3.6×10^{-4} ($E = 0.1$ J·cm^{-2}) and QE_{max}(sb) = 5.7×10^{-4} (Table 1). It is important to note

(30) (a) Tchou, J.; Bodepudi, V.; Shibutani, S.; Antoshechkin, I.; Miller, J.; Grollman; A. P.; Johnson, F. *J. Biol. Chem.* **1994**, *269*, 15318−15324. (b) Ravanat, J.-L.; Berger, M.; Boiteux, S.; Laval, J.; Cadet, J. *J. Chim. Phys.* **1993**, *90*, 871−879.

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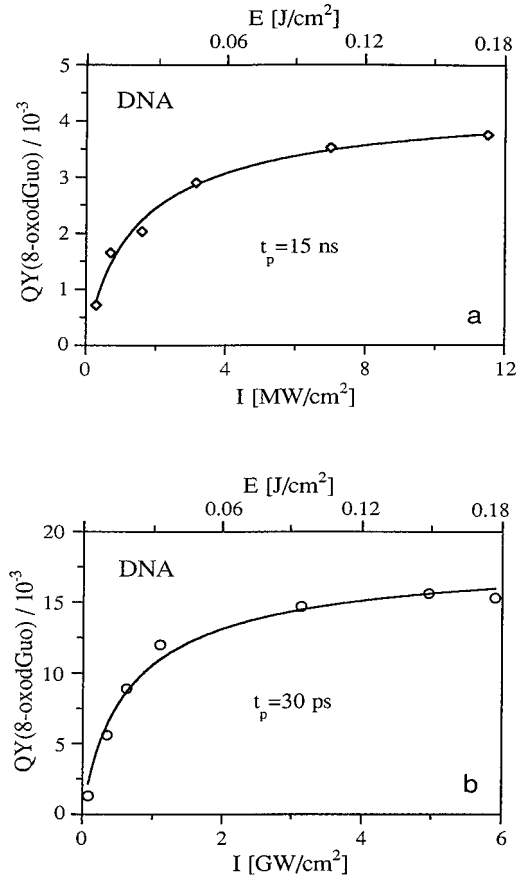

Figure 3. Laser pulse intensity (dose) dependence of the quantum yield QY on the generation of 8-oxo-dGuo upon nanosecond (a) and picosecond (b) laser photolysis of calf thymus DNA (optical thick layer, $c = 1$ mM). The solid curves represent the best fits between the experimental data and eqs 16 and 22 taken from the ref 27.

that the saturation fluence E_s for 8-oxo-dGuo is about 2-fold lower than that for the averaged value of quantum yield for DNA strand breakage (see Figure 5).

Discussion

Monomeric Purine 2′-Deoxyribonucleosides. The two major pathways of the initial transformation of the transient purine radical cations in aqueous solutions at room temperature is deprotonation and water addition.[3,5,7] It was inferred from time-resolved spectroscopy analysis that the transient radical cations of dGuo and dAdo are mainly transformed through deprotonation at neutral pH to the related oxidizing oxyl and aminyl purine radicals, respectively.[5,9,32] By analogy, this behavior was often generalized to purine bases within single- or double-stranded DNA, although direct experimental evidence was not provided.[18f,33] It should be noted that in contrast to our present data, it was recently mentioned that exposure of free dGuo to high-intensity 248 nm laser radiation leads to the formation of 8-oxo-dGuo[16] with a yield similar to that expected in DNA. However, neither quantitative data for 8-oxo-dGuo formation from dGuo nor quantum yield values for 8-oxo-dGuo within DNA were reported. In the present study, it was found that 8-oxo-dGuo and 8-oxo-dAdo which are the main hydration products of the radical cation of dGuo and dAdo, respectively, are generated as minor photoproducts with quantum yields in the 10^{-5} range. The use of •OH radical scavenger showed that only 25% of 8-oxo-dGuo and 8-oxo-dAdo are formed through the intermediary of the corresponding radical cations. In fact, the bulk of the two latter oxidized nucleosides (about 75%) is likely to be generated through initial addition of •OH radical at C8. It should be remembered that, under the experimental conditions used (optically thick samples), generation of •OH radicals through direct two-photon absorption of water is negligible upon both nanosecond and picosecond photolysis.[1,27] Interestingly, the relative contribution of •OH radicals to the quantum yields was similar irrespective of pico- and nanosecond irradiation conditions and of the laser intensity. These observations constitute additional arguments to support the hypothesis that •OH radicals originate from biphotonic excitation of dAdo and dGuo, but not from direct two-photon photolysis of water. Generation of •OH radicals upon two-step excitation of organic macrocyclic compounds to an energy level exceeding 6.5−6.8 eV was already established by spin-trapping[34] and scavenging experiments.[29] This was presumably attributed to both ionization and homolytic dissociation of photosensitized water molecules. Hydroxyl radicals are known to react efficiently with purine DNA components giving rise to the corresponding 8-oxopurine nucleosides.[3,23] It may be concluded that the contribution of •OH radicals to the biphotonic photolysis of monomeric purine 2′-deoxyribonucleosides, in terms of quantum yield, lies within the 10^{-5} range for nanosecond photolysis. An about 5-fold higher value is noted for picosecond irradiation.

The chemical reactivity of radical cations does not depend on how they are formed, and in particular of the pulse duration. Therefore, the highest value of the saturated quantum yield observed under picosecond photolysis is indicative of a higher efficiency of formation of the purine radical cation under these conditions. This demonstrates that biphotonic ionization of purines either as free nucleosides or within DNA is more efficient under picosecond excitation than upon nanosecond irradiation. This is not surprising, since it is well established that two-step excitation involves different intermediate states according to the photolysis conditions used. S_1 and T_1 excited states are generated upon exposure of purine components to pico- and nanosecond UV laser pulses, respectively.[27,28] An important matter is to determine what is the proportion of purine radical cations that is converted into 8-oxo-dGuo and 8-oxo-dAdo. It should be noted that there are no available data on the quantum yield of biphotonic ionization of monomeric dAdo and dGuo. However, the lower limit for the quantum yield for chromophore loss of free adenine and guanine upon nanosecond laser photolysis was estimated to be about $(1-2) \times 10^{-3}$.[28] Therefore, for free purine 2′-deoxyribonucleosides, the formation of 8-oxo-dGuo and 8-oxo-dAdo represents only a few percent of the overall decomposition products of the corresponding radical cations. Since 8-oxo-dGuo and 8-oxo-dAdo are formed through hydration of the related purine radical cation,[3,7,33] it may be concluded that this pathway is quite inefficient with respect to the competitive deprotonation mechanism. This is in agreement with previous observations which showed that the formation of 8-oxo-dGuo is, at the best, a minor process upon one-electron oxidation of dGuo by either type of photosensitizer[7] or $SO_4^{\bullet-}$ inorganic radical.[8] Under the former conditions, the oxazolone nucleoside whose formation arises from the deprotonation of the dGuo radical cation[10−12] is the major decomposition product.

Purine Lesions in DNA. In contrast to what was observed for free dGuo, 8-oxo-dGuo is the predominant decomposition product of the guanine radical cation within DNA. This is

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Table 1. Quantum Yields and Efficiencies of Purine Lesions and DNA Damage upon Nano- and Picosecond 266-nm Photolysis of DNA and Purine 2′-Deoxynucleosides

substrate	solvent (buffer)	lesion	laser[a]	QY ($\times 10^{-4}$)	QY_{max} ($\times 10^{-4}$)	method
dAdo	H_2O	8-oxo-dAdo	ns	0.73	0.92	HPLC-EC
			ps	3.5	5.8	
dGuo	H_2O	8-oxo-dGuo	ns	0.72	0.98	HPLC-EC
			ps	3.3	5.7	
dAdo	H_2O + 10% MeOH	8-oxo-dAdo	ns	0.16	0.2	HPLC-EC
			ps	0.9	1.4	
dGuo	H_2O + 10% MeOH	8-oxo-dGuo	ns	0.17	0.22	HPLC-EC
			ps	0.82	1.2	
ds DNA	phosphate	8-oxo-dAdo	ns[b]	14		HPLC-EC
ds DNA	phosphate	8-oxo-dGuo	ns[b]	35	45	HPLC-EC
			ps[c]	150	180	
ss DNA	phosphate	8-oxo-dGuo	ns[b]	14		HPLC-EC
ds 9G/4G	TE	8-oxo-dGuo	ns[d]	34[e]		HPLC-EC
ds 9G/4G	TE	Fpg	ns[d]	32[e]	38[e]	PAGE
ds 9G/4G	TE	ssb	ns[d]	3.6[e]	5.7[e]	PAGE

[a] The experimental error is typically less than 10%. Irradiation conditions: ns, $\tau_p = 15$ ns, $E = 0.64$ J·cm^2; ps, $\tau_p = 30$ ps, $E = 0.24$ J·cm^2; ns,[b] $\tau_p = 15$ ns, $E = 0.1$ J·cm^2; ps,[c] $\tau_p = 30$ ps, $E = 0.1$ J·cm^2; ns,[d] $\tau_p = 15$ ns, $E = 0.1$ J·cm^2. [e] QE (for the definition, see the Experimental Section.

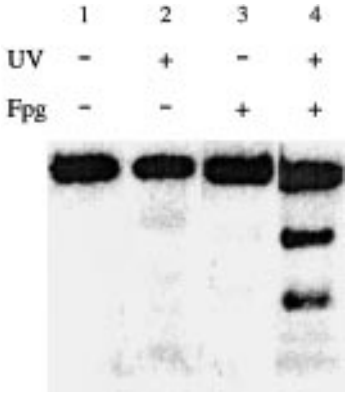

Figure 4. Denaturing gel electrophoresis of 37 bp DNA (5′-end labeled 9G strand). Nonirradiated samples: lines 1 and 3. Exposed samples to one pulse with an overall dose of 0.1 J·cm^{-2}: lines 2 and 4. Lines 1 and 2: non-Fpg-treated samples. Lines 3 and 4: Fpg-treated samples. The two strong bands in the ladder sequence on line 4 belong to the two GGG runs.

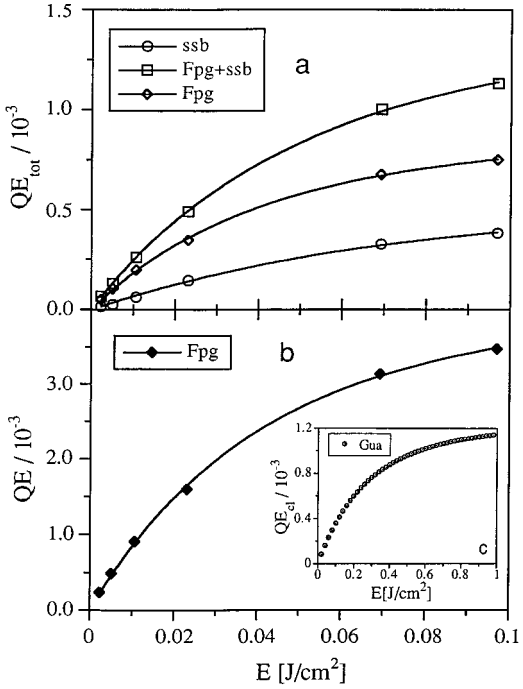

Figure 5. Laser pulse dose dependence on the quantum efficiency: (a) QE_{tot}, total photon absorbed by DNA; (b) QE, photons absorbed by guanines only. Under nanosecond laser photolysis of the 37 bp DNA (a and b) and free guanine (insert c). Single strand breaks: open circles. Fpg-sensitive lesions together with single strand breaks: open squares. Fpg-sensitive lesions only: open diamonds. Chromophore loss QE_{cl} of free guanine: insert c. The curve c was synthesized using the values of φ_1 and $\varphi_2\sigma_2$ from ref 28 and eq 2. The solid curves are the best fits between the experimental points and eq 2.

mostly inferred from a study in which riboflavin-mediated type I photosensitization was shown to efficiently generate 8-oxo-dGuo.[7] It should be added that the oxazolone photoproduct is also generated within DNA, but in a relatively lower yield than 8-oxo-dGuo.[3,10] However, the formation of both oxazolone and 8-oxo-dGuo has not been yet measured within DNA upon exposure to either nano- or picosecond laser photolysis. The observed increase in the yield of 8-oxo-dGuo in DNA cannot be unambiguously attributed to only an increase in the efficiency of the hydration pathway of the guanine radical cation. An alternative explanation could be a more efficient one-electron oxidation of the guanine residues within DNA. To answer these questions, the quantum yield for the formation of 8-oxo-dGuo in both calf thymus DNA and a 37 bp oligonucleotide was determined by HPLC-EC and further compared with the results obtained on free dGuo. Interestingly, a 100-fold increase in the yield of 8-oxo-dGuo was observed in both DNA samples as compared with free dGuo. It should be added that the value of the quantum yield for 8-oxo-dGuo in DNA is similar to that of ionization (hydrated electron generation) under the same pulse dose with $Q(e^-_{aq}) = 4.1 \times 10^{-3}$.[18c] A comparison of the yields of 8-oxo-dGuo and oxazolone in the same 37 bp duplex and in other synthetic DNA fragments showed that the former is in average twice higher.[31] Consequently, it may be concluded that 8-oxo-dGuo is a major decomposition product of the guanine radical cation in double-stranded DNA. Therefore, it appears that hydration of the guanine radical cation within DNA is a predominant process in contrast to that was observed for free dGuo. This is a direct demonstration of the crucial role played by base stacking on the chemical transformation of the primary guanine radical cation. However, an enhancement of Q(8-oxo-dGuo), although less important, is also observed in single-stranded DNA. This may be explained by the occurrence of a partial base stacking. The high efficiency of hydration of the guanine radical cation in DNA may be related to solvatation effects. It is likely that the deprotonation of the guanine radical cation in DNA is not as efficient as in free dGuo. Interestingly, recent femtosecond time resolution studies showed that the disappearance of the radical cations, at least for thymine, is likely

to be completed in less than 1 ps.[36] This would suggest that the primary transformation processes are very fast and inaccessible to usual laser flash-photolysis techniques.

Another major observation deals with the efficient formation of 8-oxo-dAdo in calf thymus DNA upon laser photolysis, showing that guanine residues are not exclusive ultimate targets of photoionization. This strongly suggests that the hole trapping ability of guanines within DNA, as proposed on the basis of a microsecond time-resolution spectroscopy analysis,[18f,32,33] and gel sequencing studies[18a] is, at least, overestimated. The presently reported data show also that the primary chemical reactions of the purine radical cations are strongly dependent on the structure in which the purine moiety is inserted. Consequently, microsecond time scale transient absorption spectra corresponding to secondary radicals arising from photoionization of either free nucleosides or single- or double-stranded DNA are likely to represent different mixtures of intermediates. It is noteworthy that conclusions based on such comparisons should be considered with care.[18f,32,33]

Another major observation was inferred from the comparative quantitative study of the formation of 8-oxo-dGuo and Fpg-sensitive sites within the 37 bp duplex DNA. It was found that QE(8-oxo-dGuo) = QE(Fpg). Therefore, it may be concluded that the overwhelming transformation product of the guanine radical cation within DNA that is cleaved by the Fpg protein is 8-oxo-dGuo. The formation of other lesions from the guanine radical cation, including the formamidopyrimidine derivative of guanine and apurinic sites which may be also recognized by the Fpg protein,[19] should be at the best minor processes. Thus, Fpg treatment followed by gel electrophoresis constitutes an almost quantitative tool for determining 8-oxo-d-Guo in photoionized DNA.

The saturation intensity in DNA is about 1 order of magnitude lower than in monomeric 2′-deoxyguanosine. The slope of the intensity response curve of the quantum efficiency for nanosecond biphotonic photolysis depends on the laser pulse dose (energy per unit area). However, this is independent of the laser pulse shape.[27,28] Therefore, it is preferable to analyze the saturation characteristics in terms of saturation pulse dose (E_s), corresponding to $QE_s = 0.63QE_{max}$,instead of saturation intensity. It is important to note that there is an almost 10-fold decrease of E_s in the case of DNA, (Figures 2 and 3). The discussion will be restricted to the data for nanosecond photolysis in optical thin layer which are more easily to be processed (see Figure 5 and refs 27 and 28). The relationship between the quantum efficiency of a biphotonic photochemical process and the laser fluence is given by the expression

$$QE = \frac{R}{\sigma_1 E_{tot}}\left[1 - \frac{e^{-\varphi_1\sigma_1 E} - e^{-\varphi_2\sigma_2 E}}{1 - \frac{\varphi_1\sigma_1}{\varphi_2\sigma_2} \quad 1 - \frac{\varphi_2\sigma_2}{\varphi_1\sigma_1}}\right] \qquad (2)$$

where R is the probability of the radical cation to form the product ($R < 1$) and σ_1 and σ_2 are the absorption cross sections of the ground singlet S_0 and excited triplet T_1 states, respectively. In addition, φ_1 and φ_2 are the quantum yields for T_1 excitation and for photoionization from T_1, respectively. In the absence of energy and charge-transfer processes, φ_1 coincides with the intersystem crossing yield whereas φ_2 corresponds to the ionization probability from the highly excited state. E_{tot} and E are the overall and the pulse irradiation doses, respectively, expressed in photons·cm^{-2}. Taking into account both, the value of $\varphi_1\sigma_1$ lying in the 5×10^{-19} to 5×10^{-20} cm^2 range[1,27,28] and the slope of the curves, approximated according to eq 2, it was found that $\varphi_2\sigma_2 \gg \varphi_1\sigma_1$. In other words, the saturation of the quantum efficiency is due to the saturation of the second transition. With a good approximation, the following relations were extrapolated from eq 1:

$$QE_{max} \approx R\varphi_1; \quad E_s \approx 1.5/\varphi_2\sigma_2 \qquad (3)$$

Thus, it is clear that the saturation dose E_s and the maximum quantum efficiency of the radical cation QE_{max} are determined by φ_2 and φ_1, respectively. Consequently, the lower value of E_s (0.045 J·cm^{-2}) that was observed for the average of guanines in DNA (Figure 5b), as compared to isolated dGuo (0.3−0.35 J·cm^{-2})[28] (Figure 5c, insert), is due to a higher value of φ_2, assuming that σ_2 does not change considerably. An important decrease in the saturation intensity for the biphotonic photolysis in double-stranded DNA as compared to single-stranded DNA and free nucleobases was already observed under picosecond laser photolysis.[37] This was attributed to a cooperative excitation due to energy migration over a rather long (150−200 bp) distance. However, the present data for nanosecond photolysis of the 37 bp DNA fragment are incompatible with such a mechanism that should require a longer energy transfer distance than the number of bases in the DNA fragment used. It is noteworthy that the cumulative energy of the two absorbed quanta largely exceeds the electron ejection threshold for all four types of nucleobases[1,18c] and they are all readily ionizable. The reason for the decrease in the saturation dose in DNA may be attributed either to an enhanced value of the ionization probability from highly excited state or to a positive charge (hole) migration[38] with a preferential trapping by guanine residues. The consequence of the former process should be also an enhancement of φ_2 for the electron donating bases and a concomitant decrease of φ_2 for the electron accepting bases. However, the considerable decrease of E_s for guanine residues in DNA, if explained only by hole transfer, should require a longer hole migration distances than that available in the 37 bp oligonucleotide. In addition, only a 2-fold higher E_s value was observed for guanine lesions as compared to the averaged value for DNA strand breaks at all sites (see below and Figure 5). Therefore, the present data are compatible with the involvement of both effects: probably a short-range (1−3 base distance) hole migration to guanines and an enhanced ionization ability of all bases in DNA as compared to free nucleosides.

DNA Strand Breakage. The quantum efficiency for strand breakage ($QE(sb) = 3.6 \times 10^{-4}$) in the 37 bp DNA duplex upon exposure to a 0.1 J·cm^{-2} fluence was inferred from electrophoresis analysis. In addition, the value for $QE_{max}(sb)$ was estimated to be 5.7×10^{-4}. The literature is abundant with values of quantum yields (efficiencies) for strand breakage of nucleic acids under nanosecond laser photolysis which are scattered within the 10^{-5}−10^{-4} range:

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(i) plasmid assay: 8×10^{-6};[39] 4.5×10^{-4};[17] $1-2 \times 10^{-4}$.[18d]
(ii) analytical centrifugation: 1.7×10^{-4} in TMV RNA.[40]
(iii) low-angle light scattering: 6×10^{-5} at $E = 0.1$ J·cm^{-2} in double-stranded and single-stranded calf thymus DNA. [2,18c]

The low yield of strand breakage and the lack of significant base specificity are characteristic of biphotonic photolysis of DNA.[15,16,31] However, somewhat higher QE(sb) values are observed using denaturing gel electrophoresis analysis. Therefore, it may be anticipated that, under the latter conditions, heat labile base lesions may lead to the formation of additional DNA strand breaks. Consequently, the values of QE(sb) presently reported should be considered as a higher estimate, since they could be attributed not only to immediate strand breaks but also to heat unstable base lesions. In this respect it should be reminded that direct frank breaks were found to be generated at the best in very low yields within DNA upon exposure to either 248-nm[16,18d] or 193-nm[18h] laser pulses. Finally, the involvement of •OH radicals under nanosecond biphotonic photolysis, although restricted within the 10^{-5} quantum yield range, was unambiguously demonstrated. The highly oxygen reactive species are likely to explain, at least partially, the formation of frank DNA strand breaks. However, it should be noted that addition of •OH scavengers was not found to exert any significant protection against strand cleavage.[2,18c] This may be explained, at least partly, by proximity effects.

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Conclusions

Evidence is provided in this work that exposure of calf thymus DNA and a synthetic duplex oligonucleotide (37-mer) to nano- and picosecond UV laser pulses gives rise to 8-oxo-7,8-dihydro-2′-deoxyguanosine and to a lesser extent to 8-oxo-7,8-dihydro-2′-deoxyadenosine. However, it should be noted that the two latter purine oxidation products are generated from the corresponding 2′-deoxyribonucleosides in a yield which is about 2 orders of magnitude lower than in DNA. This illustrates the preponderant role played by the DNA conformation features and more likely base stacking in promoting the hydroxylation of the C8 position of both purine moieties. A reasonable mechanism for the formation of both oxidation products is initial one-electron oxidation of the purine rings which is followed at least partly by hydration. The significant formation of 8-oxo-dAdo within double-stranded DNA would indicate that adenine may be a secondary sink for positive hole transfer according to the sequence context. Work is in progress by using defined sequence oligonucleotides to further evaluate this possibility.

Acknowledgment. The work was supported by the National Science Foundation (Bulgaria) (Contract F-513), French Ministry of Science and Research (ACC-SV No. 8-MESR 1995), and the CNRS (Bulgarian Academy of Sciences Collaboration Program III.1). D.A. acknowledges the University of Paris VI for providing a PAST fellowship. This paper is dedicated to Professor Waldemar Adam on the occasion of his 6oth birthday.

JA971728R