

Strand Breaks in X-Irradiated Crystalline DNA: Alternating CG Oligomers

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Razskazovskiy, Y., Debije, M. G., Howerton, S. B., Williams, L. D. and Bernhard, W. A. Strand Breaks in X-Irradiated Crystalline DNA: Alternating CG Oligomers. *Radiat. Res.* 160, 334–339 (2003).

Direct ionization of crystalline d(CGCGCGCG) and d(CGCGCGCG) oligomers produces 3'- and 5'-phosphate-terminated fragments as the main strand breakage products detectable by ion-exchange chromatography. The nature of the base has no effect on the probability of strand breakage at the given site. The yields of 3'-phosphates are systematically lower than the yields of the 5'-phosphates originating from the same cleavage site, pointing to the possible presence of unidentified products with sugar remnants attached to the 3'-end. These results show that direct ionization is efficient at producing single-strand breaks in DNA and its action is relatively indiscriminate with respect to base sequence. © 2003 by Radiation Research Society

INTRODUCTION

Direct-type effects arise from the direct ionization of the DNA molecule or from holes and electrons transferred to the DNA from its solvation shell (1–3). This pathway, resulting in direct-type damage, may be particularly important in chromosomes, where the concentration of “free” water is relatively low. Work on cellular DNA has implicated direct-type damage as a significant factor in causing strand breaks (4).

Recently we reported the yields of single-strand breaks in DNA crystals formed by d(CGCG)₂ and d(CGCGCG)₂ duplexes X-irradiated at both 4 K and room temperature (5). This study showed that the yield of strand breaks is fairly insensitive to the irradiation temperature and corresponds to about 10% of the free radical yield measured at 4 K. With respect to individual product yields, statistically sound conclusions were not possible because these two sequences were too short. One observation of particular interest was the difference in yield between products with 5'-

phosphorylated ends compared to products with 3'-ends, the former being about 1.8-fold greater than the latter. To explore this and other observations further, we have extended our study to the crystals formed by the d(CGCGCGCG) and d(CGCGCGCGCG) sequences.

Based on our previous results on unaltered base release from several DNA crystals, we hypothesized that the precursor for the strand break in directly ionized DNA is the sugar radical cation, which further undergoes deprotonation to form a neutral sugar radical (6). This process competes with hole transfer to DNA bases. Since guanine is the most oxidizable DNA base, it should “quench” the sugar radical cation more efficiently than any other base if all the processes proceed within the normal Marcus region (7). Such a correlation does exist for hole transfer from the unsaturated sugar radical cations generated from the C4'-sugar radical precursor (8). However, hole transfer from the saturated primary sugar radical cations generated through direct ionization is expected to be more exothermic, and it therefore has a chance of falling in the inverted Marcus region. If this is the case, the correlation mentioned above would not hold. The lack of site specificity of strand breaks in directly ionized crystalline DNA may be an indication that hole transfer from directly oxidized sugars to bases does proceed in this regime (9). Through the investigation of crystalline d(CGCGCGCG)₂ and d(CGCGCGCGCG)₂, the evidence continues to build toward the conclusion that base sequence has either a minor or no influence on the probability of strand breakage by direct ionization.

METHODS

Materials

The oligodeoxynucleotides, d(CGCGCGCG) (CG8) and d(CGCGCGCGCG) (CG10), were purchased from Ransom Hill Bioscience and were used as received for crystallization. Synthetic 3'- and 5'-phosphorylated oligodeoxynucleotides from Midland Certified Reagent Company were employed as reference compounds in the identification of DNA strand break products.

Crystals of CG8 and CG10 were grown following the published procedure for CG8 (10). Because the crystal structure of CG10 had not been determined previously, X-ray diffraction data were taken and analyzed by two of us (SBH and LDW). CG10 was found to be nearly isomorphous

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to CG8 (10) and to the closely related crystal of d(CGCICICG)₂ (11). In each case, the Z-DNA duplexes are packed so as to form parallel columns of continuously stacked CG base pairs. However, CG10 crystals differ by displaying disorder along the unit cell axis parallel to the columns; that is, the CG base pairs of CG10 are in phase with one another but the termini of the helices are not. While the impact of such disorder on the radiation chemistry is not known, we believe it is likely to be negligible relative to other variables.

Product Analysis

The protocol for sample preparation and irradiation has been described elsewhere (12). The crystals were irradiated with 70 keV X rays at room temperature to a required dose, transferred to 1-ml plastic vials, and weighed to $\pm 1 \mu\text{g}$ accuracy on a Cahn-60 microbalance. The crystals (samples ranged from 90–400 μg ; a typical sample weighed $\sim 200 \mu\text{g}$) were then dissolved in a 50 mM Tris/10% acetonitrile medium (pH 10.2, 50 μl of the buffer per 100 μg of crystals) containing 50 μM thymidine-5'-monophosphate (TMP) employed as an internal standard for quantification purposes and held at 70°C for 30 min (5, 6). This preventive measure was taken to ensure the absence of any uncontrolled strand breakage originating from heat-labile lesions during subsequent sample processing.

The DNA cleavage products were analyzed by ion-exchange chromatography (IEC) on a Waters Alliance[®] system equipped with an auto-sampler, a 2690 solvent delivery module, and a 996 PDA detector using a Dionex DNAPac PA-100 4.6 \times 250-mm column operated at 60°C and a flow rate of 1 ml/min. The column was washed with 50 mM Tris buffer containing 10% acetonitrile by volume (pH 10.22), and the products separated by applying a linear Cl gradient (typically from 0.1 M to 1 M NaCl over 30 min). The products were detected at 260 nm and identified by co-injection with authentic reference compounds.

Quantification

Areas under the identified product peaks (S_{pr}) were converted into the product absorbencies (A_{pr}) using the formula

$$A_{\text{pr}} = A_{\text{st}} S_{\text{pr}} / S_{\text{st}},$$

where A_{st} and S_{st} are the absorbance and the peak areas of the internal standard TMP. The typical concentration and absorbance of TMP in the loading buffer was 50 μM , corresponding to 0.33 o.d.u. at 260 nm. The areas are further converted into concentrations using the nm/o.d.u. ratios provided by Midland Certified Reagent Company for each of the references, and then into the number of micromoles of the products formed in the crystalline DNA using the known volume of the buffer used to dissolve the DNA. The absorbed energy in joules is calculated from the dose rate and the mass of the crystal. The slope of the product amount plotted as a function of dose is the radiation-chemical yield of the product expressed in $\mu\text{mol}/\text{J}$.

RESULTS

There were two experimental constraints that we were unable to overcome. One was a very limited amount of sample, due to the cost of materials and difficulties in crystallization. The other was that the presence of impurities in the starting material and the strong overlap of the peak of the parent oligomer with the peaks of some of the products in IEC chromatograms impeded quantification of the products more than 6–7 bases in length. As a consequence, the measured yields have significantly larger errors than those reported in our previous studies. But a major advantage of IEC in studies of DNA damage is that it allows for the

simultaneous detection of each strand cleavage products in one experiment. This is especially helpful when comparing product yields. Relative yields are the focus of this report.

Another advantage of IEC is that the relative retention times of end-phosphorylated DNA fragments generated from alternating CG sequences can be predicted from three simple rules. Looking at the chromatogram of CG8 in Fig. 1A, five groups of product are readily identified. Each group consists of two peaks corresponding to the products of the same length in terms of the number of bases n. Rule 1 is that the total charge on the phosphates is a key factor controlling the retention times. Therefore, any n-mer always runs faster than any n+1-mer regardless of their composition. Therefore, the group of monomers comes out before the group of dimers, dimers before trimers, etc. Rule 2 is that inside each group the cytosine-rich product always runs faster than the guanine-rich one owing to the presence of the extra negative charge on guanines at pH 10.2 (the pK_a of guanine is ~ 9.4). Rule 3 is that in sequences of the same composition and length 3' phosphates run slightly faster than 5' phosphates. These groups with even n's (such as CGp and pCG, CGCGp and pCGCG, etc.) appear as easily identifiable "doublets" in IEC chromatograms, which separate the groups corresponding to odd n's. These rules are useful when reflecting on the nature of the identified peaks, which we return to in the Discussion.

CG8

An ion exchange chromatogram of irradiated CG8 is shown in Fig. 1A. The large off-scale peaks belong to the TMP reference and the undamaged CG8. All the major peaks that grow in with increasing dose are 3'- and 5'-end phosphates; i.e., remnants of the sugar that sustained the initial damage are no longer covalently bound. None of the major products are 3'-phosphoglycolates, which are common when damage is initiated by OH radicals in oxygenated medium (13).

All the major products were identified and quantified except for CGCGCGp and pCGCGCG. The latter two were problematic, because these peaks overlap with a strong impurity peak, the intensity of which decreases with dose. These two hexamers were therefore excluded from further analysis. Amounts of the products per gram of the crystal, calculated after dissolving the crystals in the loading buffer, are reported in Table 1 along with the yields and uncertainties obtained by the linear regression analysis of the dose response curves.

CG10

The ion exchange chromatogram of CG10 irradiated with 400 kGy is shown in Fig. 1B along with the chromatogram of an unirradiated sample. Due to the superposition with intense peaks from contaminants and starting material, quantification of Cp, pGCGCGCG, the two 8-mers, and two

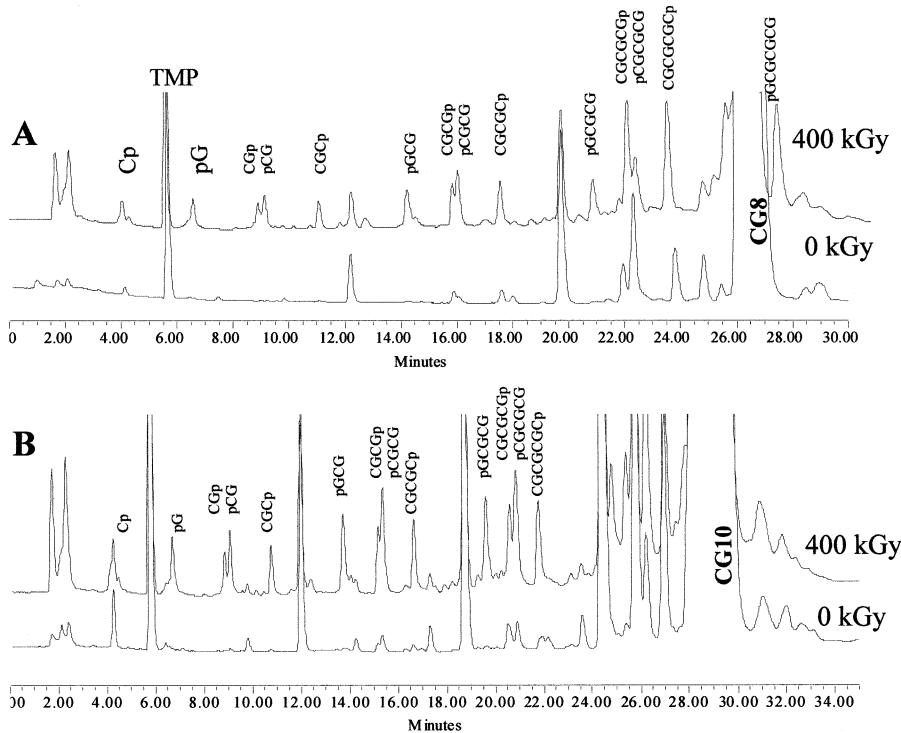


FIG. 1. Ion-exchange chromatograms of CG8 (panel A) and CG10 (panel B) before and after irradiation with 400 kGy.

9-mers was not pursued. The results for the other cleavage products are reported in Table 2.

Because the quantity of material allowed for only three samples (two irradiated + one control), the absolute values for the yields reported in Table 2 are rough estimates of the actual yields. Ratios of the yields, however, reflect relative efficiencies of product formation with reasonable accuracy.

DISCUSSION

The chromatogram of DNA cleavage products generated from CG8 and CG10 and detected by IEC is fairly simple, consisting predominantly of 3'- and 5'-phosphates. The presence of other products is likely, but they either are present in smaller quantities or have retention times comparable to that of the starting material. The latter is expected for many of the products with damage restricted to the bases. For example, the dihydropyrimidines are major products observed by Swarts *et al.* in hydrated, solid-state DNA (14, 15). However, it is highly unlikely that dihydropyrimidine-containing oligomers would be resolved from the starting material by IEC (given no difference in charge state). In addition, given the moderate alkalinity of the solutions (pH 10.2), the oligomers that sustain base damage are not likely to give rise to strand breaks. It is also notable that we do not detect oligomers containing the well known oxidation products of guanine even though one-electron oxidation of guanine most assuredly occurs at yields (15) higher than the sum of the strand break product yields.

The DNA fragmentation pattern induced by direct ionization is similar to the one induced by the indirect effect in the absence of oxygen, where hydroxyl radicals abstract hydrogen from the sugar-phosphate moiety (13). This similarity supports our model that strand breaks, produced by direct-type effects, arise from sugar radical precursors.

A notable difference between direct-type and indirect-type products is that the former contains a relative paucity of products containing a sugar remnant attached to the phosphate. Such products are well known in radiation chemistry of DNA in solution, both oxygenated and anoxic

TABLE 1
Radiation-Chemical Yields of Product Formation in Crystalline CG8

Product	Amount ($\mu\text{mol/g}$)				Yield (nmol/J)
	100 kGy	200 kGy	300 kGy	400 kGy	
Cp	1.4	1.2	1.3	4.7	9 \pm 4
pG	1.1	1.2	1.5	3.6	7 \pm 2
CGp	0.4	0.4	0.5	1.4	3 \pm 1
pCG	0.5	0.4	0.6	1.5	3 \pm 1
CGCp	0.3	0.3	0.4	1.0	2.0 \pm 0.5
pGCG	0.4	0.5	0.5	1.3	2.7 \pm 0.7
CGCGp	0.3	0.3	0.2	0.8	1.4 \pm 0.5
pCGCG	0.6	0.6	0.6	1.5	3.0 \pm 0.8
CGCGCp	0.2	0.2	0.2	0.9	1.6 \pm 0.6
pGCGCG	0.5	0.4	0.4	1.0	1.9 \pm 0.6
CGCGCGp	0.8	0.1	0.1	1.2	2 \pm 2
pGCGCGCG	1.1	0.9	1.1	2.6	5 \pm 2

TABLE 2
Radiation-Chemical Yields of Product Formation in Crystalline CG10

Product	Amount ($\mu\text{mol/g}$)		Approximate yield (nmol/J)
	100 kGy	400 kGy	
pG	2.20	4.45	17
CGp	0.50	1.31	4
pCG	0.91	1.97	7
CGCp	0.46	1.17	4
pGCG	0.79	1.98	6
CGCGp	0.28	0.85	2
pCGCG	0.89	1.99	7
CGCGCp	0.43	1.00	3
pGCGCG	0.71	1.66	6
CGCGCGp	0.39	0.83	3
pGCGCG	0.82	1.58	6
CGCGCGp	0.59	0.95	4

(13, 16). The structure of the sugar remnants vary: Three such structures have been proposed for the remnants attached at the 3'-end, and two for the 5'-end (16). While we find that the dominant reaction pathway for direct strand breakage is for the damaged sugar to degrade with the release of the 3'- and 5'-phosphates and the free base, we argue below that the yield of products with attached sugar remnants is likely to be significant as well.

The yield of a 5'-phosphate is systematically higher than the yield of a 3'-phosphate originating from the same cleavage site. This same phenomenon occurs in crystalline d(CGCG)₂, d(CGCGCG)₂, d(CACGCG):d(CGCGAT), and d(CGCACG):d(CGTGCG) (5, 9). (However, in these shorter sequences this effect is partially obscured by the increased probability of damage to the terminal sugars.) The number of reasonable explanations for this imbalance is limited. We suggest that products still carrying sugar remnants attached to the terminal phosphates are formed, with the yield of products containing 3'-attachments greater than the yield of those containing 5'-attachments. A number of factors make these products more difficult to identify and quantify: variability in remnant structures, lower yields, and lack of reference compounds. If this explanation is correct, the yields of such products containing remnants must be greater than or equal to the difference between the 5'-phosphate and 3'-phosphate products. The IEC data are consistent with this explanation.

Material balance is expected between the products on one side of the cleavage site and those on the other side of the cleavage site. The IEC data would be consistent with this expectation if they contain unidentified peaks with retention times in the ranges expected for products containing sugar remnants. The retention time of a product containing a sugar remnant can be estimated using the rules outlined above. As an example, consider damage at C5 of CG8; Fig. 2 shows that the yields of the two major products, CGCGp and pGCG, differ by about a factor of two. This difference would be balanced out if the yield of CGCGpR exceeds

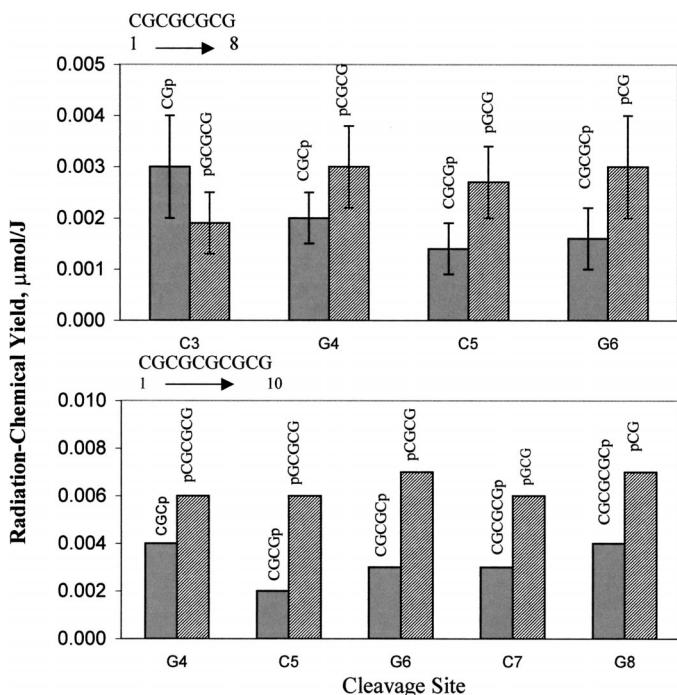


FIG. 2. Product yields in CG8 and CG10 grouped by cleavage site. Site numbering starts at the 5'-end.

the yield of RpGCG (where R is a sugar remnant) by a compensating amount. Since the excess of CGCGpR would need to be comparable to the yield of CGCGp, one would expect to see peaks attributable to CGCGpR, if the structural variety of R is limited. The retention time of CGCGpR can be estimated using the rules outlined above. It should fall on the slow side of the 3-mer group because the sum of phosphate charges is -4. The expected retention time is therefore close to that of pGCG (14.3 min). Indeed, at 14.4 min, there is an unidentified peak that grows in with dose. If one goes through this exercise with each of the product pairs shown in Fig. 2, one finds unassigned product peaks that could correspond to the respective 3'-phosphate product with a remnant attached. Hopefully, this hypothesis will be tested; this would be straightforward if sufficient product, in the unidentified peaks, can be isolated for further analysis.

Another observation with respect to relative yields is that the yields for Cp and pG, in both CG8 and CG10, are two to four times that of the other products listed in Tables 1 and 2. The level of error in these two measurements, however, is large. The peak shapes indicate a superposition of each mononucleotide with unidentified products that have similar retention times and comparable intensity. The low extinction coefficients of Cp and pG, relative to the other cleavage products, make their quantification especially error-prone. For this reason, these two products were excluded from the analysis given in Tables 3 and 4.

Tables 3 and 4 show radiation-chemical yields for products in CG8 and CG10 grouped according to the cleavage site they originate from. Figure 2 shows the product yields

TABLE 3
Radiation-Chemical Yields of Product Formation in CG8 Grouped by the Cleavage Site

Site	Product	Radiation-chemical yields (mmol/J)					
		5'-side		3'-side			
		Yield	Error	Product	Yield	Error	Average
C3	CGp	0.0030	0.0010	pGCGCG	0.0019	0.0006	0.0025
G4	CGCp	0.0020	0.0005	pCGCG	0.0030	0.0008	0.0025
C5	CGCGp	0.0014	0.0005	pGCG	0.0027	0.0007	0.0020
G6	CGCGCp	0.0016	0.0006	pCG	0.0030	0.0010	0.0023

Note. Site numbering starts at the 5'-end.

from these tables plotted as a function of cleavage site for all more or less reliably quantifiable products in CG8 and CG10. Based on average yields, the cleavage probabilities appear to be fairly uniform for all internal sites regardless of the base. This is consistent with the model in which the rate of hole transfer from the sugar to the base is not controlled to any significant extent by the oxidation potential of the base as it should be for a highly energetic process (9). It is also consistent with our earlier results on unaltered base release from crystalline DNA that showed a statistically equal probability of damage for all sugars in the sequence.

A quantity of considerable interest is the yields of immediate strand breaks. In our previous studies of strand break products in crystalline DNA (5, 9), the yields (in $\mu\text{mol}/\text{J}$) are 0.14 ± 0.03 for d(CGCG)₂, $0.06-0.11$ for d(CGCGCG)₂, 0.16 ± 0.03 for d(CGCACG):d(CGTGCG), and 0.07 ± 0.02 for d(CACGCG):d(CGCGTG). Stressing the above caveats regarding absolute accuracy and using extrapolated values for the products that could not be quantified, the strand break yields for CG8 and CG10 are in the ranges $0.02-0.04 \mu\text{mol}/\text{J}$ and $0.03-0.08 \mu\text{mol}/\text{J}$, respectively. These approximate yields continue to build the case for $\sim 10\%$ of the direct ionizations resulting in immediate strand breaks and that the break is initiated by one-electron oxidation of the sugar phosphate backbone.

CONCLUSIONS

The rate of electron transfer from DNA bases to the holes generated on the sugar-phosphate backbone in directly ion-

ized DNA is not controlled by the nature of the base, in agreement with the large negative ΔG expected for the process. The sugar damage results in a strand break with release of a pair of 3'- and 5'-end phosphates per each cleavage site. The observed preference for the production of 5'-phosphorylated products suggests that some sugar remnants remain attached to the terminal phosphates primarily at the 3'-end. Products containing sugar remnants may account for IEC peaks that have not yet been identified.

ACKNOWLEDGMENTS

We thank Kermit R. Mercer for his invaluable technical assistance. The investigation was supported by PHS Grant 2-R01-CA32546, awarded by the National Cancer Institute, DHHS. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

Received: February 7, 2003; accepted: April 12, 2003

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TABLE 4
Radiation-Chemical Yields of Product Formation in CG10 Grouped by the Cleavage Site

Site	Product	Radiation-chemical yield (mmol/J)					
		5'-side		3'-side		Yield	Average
		Yield	Product	Yield	Product		
G4	CGCp	0.004	pCGCGCG	0.006	pCGCG	0.005	
C5	CGCGp	0.002	pGCGCG	0.006	pGCG	0.004	
G6	CGCGCp	0.003	pCGCG	0.007	pGCG	0.005	
C7	CGCGCGp	0.003	pGCG	0.006	pGCG	0.004	
G8	CGCGCGCp	0.004	pGCG	0.007	pGCG	0.006	

Note. Site numbering starts at the 5'-end.

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