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Mechanisms of Strand Break Formation in DNA due to the Direct Effect of Ionizing Radiation: The Dependency of Free Base Release on the Length of Alternating CG Oligodeoxynucleotides

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Abstract

The question of how NA base sequence influences the yield of DNA strand breaks produced by the direct effect of ionizing radiation was investigated in a series of oligodeoxynucleotides of the form $(d(CG)_n)_2$ and $(d(GC)_n)_2$. The yields of free base release from X-irradiated DNA films containing 2.5 waters/nucleotide were measured by HPLC as a function of oligomer length. For $(d(CG)_n)_2$, the ratio of the Gua yield to Cyt yield, R , was relatively constant at 2.4–2.5 for $n = 2–4$ and it decreased to 1.2 as n increased from 5 to 10. When Gua was moved to the 5' end, for example going from $d(CG)_5$ to $d(GC)_5$, R dropped from 1.9 ± 0.1 to 1.1 ± 0.1 . These effects are poorly described if the chemistry at the oligomer ends is assumed to be independent of the remainder of the oligomer. A mathematical model incorporating charge transfer through the base stack was derived to explain these effects. In addition, EPR was used to measure the yield of trapped-deoxyribose radicals at 4 K following X-irradiation at 4 K. The yield of free base release was substantially greater, by 50–100 nmol/J, than the yield of trapped-deoxyribose radicals. Therefore, a large fraction of free base release stems from a nonradical intermediate. For this intermediate, a deoxyribose carbocation formed by two one-electron oxidations is proposed. This reaction pathway requires that the hole (electron loss site) transfers through the base stack and, upon encountering a deoxyribose hole, oxidizes that site to form a deoxyribose carbocation. This reaction mechanism provides a consistent way of explaining both the absence of trapped radical intermediates and the unusual dependence of free base release on oligomer length.

Introduction

Biological responses to ionizing radiation are rooted primarily in the damage produced in DNA.^{1,2} Two types of damage occur: direct-type due to energy deposited in DNA itself or in the first solvation layer of DNA and indirect-type where energy deposited in the bulk water creates hydroxyl radicals and aqueous electrons that react with DNA. In vivo, the contribution of direct-type damage is estimated to be comparable to that of indirect-type damage.^{3,4} The work reported here is part of an ongoing effort to determine the chemical mechanism for a class of damage known as strand breaks (sb), focusing on sb produced by the direct effect.

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Strand breaks in high molecular weight DNA have been measured using a number of different approaches.^{5–8} With regard to determining the chemical mechanism, these approaches are limited by difficulties in correlating the breakage site with the base sequence at that site. This limitation is lifted if sb are measured in oligodeoxynucleotides of known sequence, which has been done for 8 different oligomers.^{9–12} However, these experiments, using ion exchange HPLC to quantify the actual sb products, were limited to relatively short oligomers having simple sequences because of the need to achieve sufficient resolution of the product peaks. A way to circumvent this problem is to measure free base release (fbr) as an indicator of strand breaks.¹³ While it is not yet certain that there is a 1:1 correlation between fbr and sb for the direct effect, we consider this to be a good operating assumption.¹⁴

Our recent study on fbr in the d(CGCGCG)₂ hexamer¹⁵ gave the surprising result that the chemical yield of guanine, $G(\text{Gua})$, was 2-fold greater than the yield of cytosine, $G(\text{Cyt})$. The fbr yields and their ratio, $R = G(\text{Gua})/G(\text{Cyt})$, were relatively independent of whether the samples were prepared as films or crystals and whether the samples were irradiated at RT or at 4 K followed by warming to RT. Over this range of conditions, the yield of total free base, $G(\text{fbr})$, was between 112 and 126 nmol/J. Furthermore, $G(\text{fbr})$ was significantly larger than the yield of radicals trapped on deoxyribose, $G_{\text{dRib}}(\text{fr})$, which was between 40 and 90 nmol/J. Because dRib radicals are known precursors to fbr, the observation that $G_{\text{dRib}}(\text{fr})$ was less than $G(\text{fbr})$ required the presence of a nonradical intermediate. These two findings, $R > 2$ and $G(\text{fbr}) > G_{\text{dRib}}(\text{fr})$, lead us to propose that deoxyribose damage is influenced by the base attached to it and/or the surrounding base sequence and that this influence is related to the formation of a nonradical intermediate by a reaction pathway that entails two one-electron oxidations. That pathway, presented in more detail below, depends on diffusion of a radical cation through the DNA base stack and, thereby, suggests that fbr should depend on the oligomer length.

The aim of the work presented here was to explore the dependency of $G(\text{Gua})$, $G(\text{Cyt})$, and R on oligomer length and use that information to better understand the mechanism by which base release is influenced by base sequence of the DNA.

Materials and Methods

Desalted synthetic palindromic oligodeoxynucleotides were obtained from Ransom Hill Bioscience and IDT. All of the oligomers, d(CG)_{*n*} (*n* = 2,3,4,5,6,7,10), d(GC)₅, d(GC)₆, d(GCGGGCCCCG), and d(GCACGCGTGC), are written 5' to 3' and both ends are phosphate free, for example d(CG)₆ ≡ d(5'-CpGpCpGpCpG-3'). Highly polymerized-lyophilized genomic DNA isolated from the bacterium *Micrococcus luteus/lysodeikticus* with 72% GC content and containing 2–4% protein were obtained from Sigma-Aldrich. All of the samples were used without further purification. OmniPur Nuclease-free water was from EM Science, Merck. DNA bases used as references for HPLC experiments were from Sigma-Aldrich. All other chemicals and solvents were Baker Analyzed and used as received from Mallinckrodt Baker Inc.

The methodology was described in detail previously,^{15,16} therefore, only a brief outline is given here. Oligodeoxynucleotide and DNA films were prepared at room temperature by slow evaporation of 10–20 μg/μL solutions on a Teflon Petri dish or within silylated suprasil quartz tubes. The dish or tubes were sealed inside a jar containing saturated NaOH solution, which maintains a relative humidity of 8%.¹⁷ After 2 to 3 weeks at this humidity, the DNA hydration level (Γ) reached a steady state and was assumed to be 2.5 mol water/mol nucleotide. The films weighed 150–250 μg, measured to an accuracy of ±1 μg, and the film mass was 70–80% DNA (including the mass of its counterions and 2.5 waters/nucleotide) with the remaining mass due to excess salt (ammonium acetate). For each film, the DNA target mass was determined to better than ±1%.

The films, held in small plastic vials under air, were X-irradiated using a Varian/Eimac OEG-76H tungsten-target tube operated at 70 keV and 20 mA. The X-ray beam was filtered by a 1 mm thick aluminum foil and by the use of the sample container wall. Dose rate calibrations were done using radiochromic film purchased from Far West Technology, Inc. For HPLC, the dose rate at the sample was 1.1 kGy/min and, for EPR, it was 0.4 kGy/min.

HPLC measurements were made on solutions prepared under air by dissolving DNA films in nuclease-free water at a mass ratio of 1:1. The pH 6.8 solutions were injected into a Waters Alliance HPLC equipped with a 2690 solvent module and 996 PDA detector. Free bases were separated on a Phenomenex Columbus C-18 reverse-phase column at 303 K. Detection was at 254 nm and quantification was by comparison with uracil as an internal standard.

EPR measurements were made at 4 K after X-irradiation at 4 K using a Janis Dewar EPR accessory¹⁸ mounted in a Varian E-12 spectrometer operating at Q-band (35 GHz) microwave frequencies. Free-radical concentrations were determined using a ruby standard mounted on the inside wall of the Q-band cavity.

Chemical yields were based on the DNA target mass, which was the DNA mass plus 2.5 waters and 1 NH_4^+ per phosphate.

Results

Free Base Release

The yields of free base release (fbr) were measured in films of oligodeoxynucleotides of the sequence $\text{d}(\text{CG})_n$, where $n = 2, 3, 4, 5, 6, 7$, and 10. The films were prepared so as to strongly favor the duplex form, $(\text{d}(\text{CG})_n)_2$ and the hydration level for all samples was $\Gamma = 2.5 \text{ mol H}_2\text{O}$ per mol nucleotide. In each case, the HPLC profiles were comparable to those published previously for $\text{d}((\text{CG})_3)_2$.¹⁵ Typical dose response curves are shown in Figure 1 for $\text{d}(\text{CG})_5$. As can be seen from the yields in Table 1, fbr showed a systematic dependence on duplex length.

The ratio for the yields of Gua to Cyt, $R = G(\text{Gua})/G(\text{Cyt})$, was found to be relatively constant (2.3–2.5) for the shorter oligomers of lengths $n = 2, 3$, and 4. R began to decrease at $n = 5$ and dropped to 1.2 at $n = 10$. The yield of total base release, $G(\text{fbr})$, decreased over this same range, from $177 \pm 9 \text{ nmol/J}$ for $n = 2$ down to $104 \pm 9 \text{ nmol/J}$ for $n = 10$. The decrease in $G(\text{fbr})$ with increasing length suggests an end effect in which the terminal deoxyribose is more labile than the internal deoxyribose. An end effect may also explain the inequality, $G(\text{Gua}) > G(\text{Cyt})$, particularly for the shorter oligomers.

The $G(\text{Gua}) > G(\text{Cyt})$ inequality in $\text{d}(\text{CG})_n$ sequences could be caused by (i) the differences in chemical structure of the deoxyribose at the C3' and C5' ends or by (ii) the deoxyribose of guanine being more prone to damage than the deoxyribose of cytosine. If (ii) and only (ii) contributes to this effect, then R should be insensitive to switching Gua and Cyt between the two ends. To test this, we measured fbr yields in $\text{d}((\text{GC})_5)_2$ and $\text{d}((\text{GC})_6)_2$, which places Gua at the 5' end and Cyt at the 3' end. The large decrease in R , for example from 1.9 to 1.1 for $n = 5$, demonstrates that (i) plays a role, that is Gua release is less likely when moved from the 3' end to the 5' end and Cyt release is more likely when moved to the 3' end. At the same time, it is important to note that the ratio did not decrease to $1/1.9 = 0.53$, which would be expected if (i), and only (i), contributes. These results indicate that the C3'–OH terminated deoxyribose is inherently more labile than the C5'–OH deoxyribose and that, at whichever end Gua is, it sensitizes the deoxyribose at that end.

Two variations of the $d(GC)_5$ sequence were explored, $d(GCGGGCCCGC)_2$ and $d(GCACGCGTGC)_2$. Both of these duplexes are A-form DNA and both share the relevant property of containing Gua at the 5' end and Cyt at the 3' end.^{19,20} We found that R was effectively the same for all three decamers, which is consistent with the oligomer ends asserting a predominant influence on R . To render the end effect negligible, high molecular weight DNA rich in Gua/Cyt was studied. *M. luteus* DNA, which contains 72% GC, gave a $G(\text{Gua})/G(\text{Cyt})$ ratio of 1.00 ± 0.10 . Taking care to note that this is the average over many different sequences within *M. luteus*, this is evidence that, in GC rich DNA, the deoxyribose of Cyt and Gua are damaged with equivalent average yields. Furthermore, the presence of low levels of Ade:Thy base pairs (<30%) did not have a measurable impact on the average for the release of either Cyt or Gua.

The yields of Cyt and Gua for $d((CG)_n)_2$, $d((GC)_5)_2$, $d((GC)_6)_2$, and *M. luteus* are plotted as a function of the number of base pairs, N ($N = 2n$), as shown in Figure 2. The dip in $G(\text{Cyt})$ (shown as solid circles) that occurs at $N = 6-10$ is a striking feature of this data, a feature that proved quite helpful in discriminating between possible mechanistic models.

Trapped Free Radicals

The chemical yield of free radicals trapped on DNA at 4 K was measured for the DNA sequences. EPR spectra of $(d(CG)_5)_2$ films irradiated at three different doses are shown in Figure 3. The intensity of the broad outer wings, relative to the narrower central signal, increased with dose and was readily visible at doses >50 kGy. By collecting data out to very high doses, it was possible to partition the DNA-trapped radicals into two types: base radicals and deoxyribose radicals.²¹ High-dose data was collected for $(d(CG)_5)_2$, making it possible to measure the yield of radicals trapped by the bases, $G_{\text{base}}(\text{fr})$, and by deoxyribose, $G_{\text{dRib}}(\text{fr})$. The initial slope of the high-dose curve in Figure 4 is the yield of total radicals, $G\Sigma(\text{fr})$, which equals $G_{\text{base}}(\text{fr}) + G_{\text{dRib}}(\text{fr})$.

For $(d(CG)_5)_2$, the measured values of $G\Sigma(\text{fr})$ and $G_{\text{dRib}}(\text{fr})$ are given in Table 2. For the other four sequences given in Table 2, data was taken only at low dose. Whereas the slope at low dose gave an accurate measure of $G\Sigma(\text{fr})$, the value of $G_{\text{dRib}}(\text{fr})$ had to be calculated under the assumption that partitioning was in the same range as observed previously in related DNA sequences. The calculated range of $G_{\text{dRib}}(\text{fr})$ for these four sequences is given in Table 2. In all five samples, we find that the yield of fbr exceeds the yield of deoxyribose trapped radicals ($G(\text{fbr}) > G_{\text{dRib}}(\text{fr})$). This observation is relevant to the free base release model proposed below because it requires that a large fraction of free base release be derived from precursors that are not trappable free radicals.

Model

The above results showed that $R = G(\text{Gua})/G(\text{Cyt})$ is not a simple function of oligomer length. A viable model needs to explain the flat response in the range $N = 4-8$, the corresponding dip in $G(\text{Cyt})$ at $N = 6-10$, and the decrease in R when the $d(CG)_n$ sequence is reversed to $d(GC)_n$. To do this, we propose a reaction scheme, derive a mathematical model based on the scheme, and apply the model to the free base release data.

Reaction Scheme

The model presented in Scheme 1 follows the reaction scheme presented previously to explain product yields in $d(GCACGCGTGC)_2$ films.¹² The scheme has two primary branches: one is a free-radical pathway initiated by one-electron oxidation (1) and the other is a molecular pathway (2). Reaction 1 requires two steps, ionization of the DNA backbone producing a radical cation followed by rapid deprotonation of the radical cation to form an uncharged carbon-

centered radical. This radical is designated as $\text{dRib}(\text{Cn}'\text{-H})^\bullet$, where Cn' is the site of hydrogen loss. (In Scheme 1, $\text{Cn}' = \text{C3}'$). In reaction **1**, there is also a back reaction due to electron return to $\text{dRib}(\text{Cn}'\text{-H})^\bullet$ followed by reprotonation. Reaction **2** is a spur reaction where the carbocation, $\text{dRib}(\text{Cn}'\text{-H})^+$, is formed in the absence of a trappable-free-radical intermediate. The carbocation is also formed via one-electron oxidation of $\text{dRib}(\text{Cn}'\text{-H})^\bullet$, a trappable intermediate, as shown in **3** using the specific example of $\text{dRib}(\text{C3}'\text{-H})^+$; this is reversible by electron addition to $\text{dRib}(\text{C3}'\text{-H})^+$. At high doses, $> \sim 10$ kGy, reactions due to multiple track interactions need to be considered. Reaction **4** is due to electron return to $\text{dRib}(\text{Cn}'\text{-H})^\bullet$ where the electron is generated by a different track than that giving rise to **1**. When the films are dissolved in water, both $\text{dRib}(\text{Cn}'\text{-H})^\bullet$ and $\text{dRib}(\text{Cn}'\text{-H})^+$ react to give free base release, **5** and **6**, respectively. Note that reactions **1–4** are driven by dose, whereas reactions **5–6** are dependent on time following dissolution.

On the basis of our earlier derivation,¹² the chemical yield of free base release is given by $G(\text{fbr}) = k_1 + k_2$, the slope of dose response curve at zero dose. In Scheme 1, k_1 corresponds to reaction **1**, k_2 to reaction **2**, and so forth. The slope of the dose response curve at high dose is given by $S_{\text{high}}(\text{fbr}) = k_2 + k_1 \times k_3/(k_3 + k_4)$. When $k_3 \gg k_4$, as expected for fbr, $S_{\text{high}}(\text{fbr}) = k_2 + k_1 = G(\text{fbr})$. Thus, $S_{\text{high}}(\text{fbr}) \approx G(\text{fbr})$, explaining why the dose response curve for fbr is, to a good approximation, linear out to unusually high doses.

The fbr release data shows that the chemistry is different for a deoxyribose at the 5' and 3' ends compared to the internal positions. We propose that this is due to reactions that proceed via alkoxy radical intermediates that can only occur if there is an alcohol function present. This is shown in Scheme 2 using the 3' end as an example. Reactions **7**, **11**, **10**, and **12** in Scheme 2 correspond respectively to reactions **1** (second step), **3**, **5**, and **6** in Scheme 1. It is the addition of reactions **8** and **9**, proceeding via the alkoxy radical, $3'\text{dRib}(\text{O3}'\text{-H})^\bullet$, that opens up an alternative pathway for formation of $3'\text{dRib}(\text{C3}'\text{-H})^\bullet$. It is known from EPR studies²² that formation of alkoxy radicals in crystalline nucleosides, nucleotides, and other carbohydrates is common and that, whereas these radicals can be trapped at 4–77 K, at higher temperatures they react to produce carbon centered radicals via H transfer or H abstraction. A key element of our model is, thereby, that formation of the $\text{dRib}(\text{Cn}'\text{-H})^\bullet$ intermediate is more probable at the oligomer ends than at internal positions.

Another key element is that the second one-electron oxidation shown as reaction **3** in Scheme 1 is due to a base radical cation, also shown in reaction **11** of Scheme 2. Ionizing radiation generates holes (sites of electron loss) on the backbone and base stack with nearly equal probability.²³ For reaction **11** to occur, a hole must first be trapped by creating a $\text{dRib}(\text{Cn}'\text{-H})^\bullet$ radical and then a base-centered hole must move into close enough proximity to oxidize the $\text{dRib}(\text{Cn}'\text{-H})^\bullet$ radical. The probability of this occurring is enhanced by 1D diffusion of the hole through the base stack as depicted in Scheme 3.

This reaction pathway, entailing two one-electron oxidations, introduces three important attributes to the model. i) The probability of two holes being formed on one oligomer must be a function of the length of the oligomer; as the length increases, the probability of intercepting two holes produced by a single track increases. ii) The probability of an encounter between a deoxyribose radical and a hole on the base stack depends on the diffusion distance of the hole through the base stack. iii) Oxidation of deoxyribose radicals by diffusing holes provides a mechanism for G–C selectivity because the holes preferentially hop between guanines, conferring a greater probability of a dRib radical being oxidized if the dRib moiety is attached to Gua. Therefore, Gua release would be more probable than Cyt release.

Mathematical Description of the Model

A function $Y(N)$ is used to describe the yield of free base by oligomers of the type $(d(CG)_n)_2$, where the independent variable N is the number of base pairs, $2 \times n$. $Y(N)$ is the sum of the yield of cytosine, $Y_c(N)$, and the yield of guanine, $Y_g(N)$. The subscripts c and g here, and throughout, refer to cytosine and guanine, respectively.

$$Y(N) = Y_c(N) + Y_g(N) \quad (1)$$

There are two mechanistically distinct parts to the model: $D(N)$, which describes free base release that depends on 1D migration of holes through the base stack (as depicted in Scheme 3) and $B(N)$, which describes free base release due to all other reaction pathways. The yield is expressed as the sum of these two parts.

$$\begin{aligned} Y(N) &= B(N) + D(N) & Y_c(N) &= B_c(N) + D_c(N) \\ \text{and} \quad Y_g(N) &= B_g(N) + D_g(N) \end{aligned} \quad (2)$$

Each part is a simple sum of the contribution from the two bases.

$$B(N) = B_c(N) + B_g(N) \quad \text{and} \quad D(N) = D_c(N) + D_g(N) \quad (3)$$

(i) Part B—Part B encompasses all events that lead to fbr that do not depend on the range of the hole as it diffuses through the base stack. The intermediates are produced by either single (reaction 1) or double oxidation (reaction 2 or 3); these oxidations may occur directly in the oligomer that ultimately gives rise to fbr or they may be transferred to that oligomer from other sites in the matrix. Double oxidation, in this case, occurs when a second hole is created at a site in the DNA matrix that lies close enough to the deoxyribose hole to permit hole–hole combination reactions but it *excludes* those combination reactions facilitated by hole diffusion through the oligomer base stack (Scheme 3).

The increased probability of fbr at the 5' and 3' ends relative to internal positions is expressed by

$$m5 = \frac{p(1)}{p(i)} \quad \text{and} \quad m3 = \frac{p(N)}{p(i)} \quad (4)$$

where the probability of fbr is $p(i)$ at any internal position i , $p(1)$ at the 5' end, and $p(N)$ at the 3' end. By definition $m5$ and $m3$ are ≥ 1.0 , $m5$ enhances Cyt release, and $m3$ enhances Gua release for this base sequence (Cyt is at the 5' end and Gua at the 3' end). The impact of this end effect decreases as N increases, approaching an asymptotic value of B_k .

$$\begin{aligned} B(N) &= B_k \frac{(m5 + m3 + N - 2)}{N} \\ &= B_k \left\{ \frac{(m5 - 1 + N/2) + (m3 - 1 + (N/2))}{N} \right\} \end{aligned} \quad (5)$$

Given that half of the bases are Cyt and the other half are Gua, $B(N)$ can be expressed as the sum of $B_c(N)$ and $B_g(N)$.

$$B_c(N) = B_c k \frac{(m5 - 1 + (N/2))}{N} = B_c k \left\{ \frac{(m5 - 1)}{N} + \frac{1}{2} \right\} \quad (6)$$

$$B_g(N) = B_g k \frac{(m3 - 1 + (N/2))}{N} = B_g k \left\{ \frac{(m3 - 1)}{N} + \frac{1}{2} \right\} \quad (6b)$$

At large N , these hyperbolic functions approach an asymptotic value of $B_c k/2$ for Cyt and $B_g k/2$ for Gua, where $Bk = 1/2 (B_c k + B_g k)$. If these equations were sufficient to describe fbr, then the ratio of the yields would be

$$R_t(N) = \frac{B_g(N)}{B_c(N)} \quad (7)$$

(ii) Part D—Part D describes the contribution made by hole diffusion through the oligomer base stack, which increases the yield of double oxidation as shown by reaction **11** in Scheme 2. An analytical solution to the steady-state problem of 1D diffusion of a hole through the DNA base stack has been presented previously.²⁴ A similar approach was applied to hole scavenging by a dRib(Cn'-H)[•] radical residing in the i th position of an oligomer of length N . As before, we assumed that the holes are injected randomly, that once injected they cannot leave the strand, and that they react with the dRib(Cn'-H)[•] trap on the first encounter. The fraction of injected holes reaching the trap, $f(i)$, was calculated as a net flux of holes to the trap divided by the rate of hole injection and multiplied by the probability $p(i)$ of finding the trap in the i th position. The net flux is defined as a sum of fluxes in oligonucleotide segments i and $N - i$ base pairs long, each terminated by dRib(Cn'-H)[•] at one end. For each of the segments, the above assumptions result in the diffusion formalism described earlier²³ with an ultimate hole sink at the dRib(Cn'-H)[•]-terminated end, and a totally reflective opposite end. The solution can be expressed in the form

$$f(i) = p(i) \frac{1}{\alpha^2 N} \ln \left(\frac{\cosh(\alpha i) \cosh[\alpha(N - i + 1)]}{\cosh[\alpha(i - 1)] \cosh[\alpha(N - i)]} \right) \quad (8)$$

where $i = 1 \dots N$, $\alpha = (2k_r/k)^{1/2}$, k_r is the rate of hole loss due to competing processes, and k is the rate of base-to-base hopping. The probability $p(i)$ is

$$p(1) = \frac{m5}{m5 + m3 - 2 + N} \quad p(N) = \frac{m3}{m5 + m3 - 2 + N} \\ \text{and } p(i \neq 1, N) = \frac{1}{m5 + m3 - 2 + N} \quad (9)$$

The sum over $f(i)$ for all N positions describes the total fbr, $F(N)$.

$$F(N) = \sum_{i=1, N} f(i) = \left(\frac{2 \ln \{ \cosh(\alpha N) \cosh(\alpha) \operatorname{sech}[\alpha(N-1)] \} + (m5+m3) \{ \cosh(\alpha N) \cosh(\alpha) \operatorname{sech}[\alpha(N-1)] \}}{\alpha^2 (m5+m3-2+N)N} \right) \quad (10)$$

This can be written in more compact form using

$$\begin{aligned} Q1 &= 2 \ln \{ \cosh(\alpha N) \cosh(\alpha) \operatorname{sech}[\alpha(N-1)] \} \\ Q2 &= \{ \cosh(\alpha N) \cosh(\alpha) \operatorname{sech}[\alpha(N-1)] \} \end{aligned} \quad (11)$$

and

$$V = \alpha^2 (m5+m3-2+N)N \quad (12)$$

Substituting eqs 11 and 12 into 10 gives

$$\begin{aligned} F(N) &= \frac{2Q1+(m5+m3)Q2}{V} = F_c(N) + F_g(N) \\ &= \frac{Q1+m5Q2}{V} + \frac{Q1+m3Q2}{V} \end{aligned} \quad (13)$$

where $F_c(N)$ and $F_g(N)$ partition $F(N)$ into functions describing Cyt and Gua release, respectively.

To apply $F_c(N)$ and $F_g(N)$ to our data, each of these functions must be multiplied by a scaling factor that reflects the fbr yield due to diffusion, $D_c k$ for Cyt and $D_g k$ for Gua. The yield due to diffusion, $D(N)$, is then treated as a simple sum of the contributions from Cyt and Gua, $D_c(N) + D_g(N)$. Both of these are weighted by a function $E(N)$ that describes the probability that a single oligomer will intercept two holes.

$$\begin{aligned} D_c(N) &= E(N) D_c k \left\{ \frac{Q1+m5Q2}{V} \right\} \text{ and} \\ D_g(N) &= E(N) D_g k \left\{ \frac{Q1+m3Q2}{V} \right\} \end{aligned} \quad (14)$$

$E(N)$ is chosen to describe the probability of a rod with length N intercepting two holes in one cluster of ionizations. The distribution of holes in the cluster is assumed to be Gaussian with a width w and centered at a distance d along the oligomer. The probability of intercepting two holes is the integral of this function, known as the error function, erf. The equations describing the diffusion dependent yields are then

$$\begin{aligned} D_c(N) &= D_c k \frac{\left\{ \operatorname{erf} \left[\frac{(N-d)}{w} \right] + 1 \right\}}{2} \left\{ \frac{Q1+m5Q2}{V} \right\} \text{ and} \\ D_g(N) &= D_g k \frac{\left\{ \operatorname{erf} \left[\frac{(N-d)}{w} \right] + 1 \right\}}{2} \left\{ \frac{Q1+m3Q2}{V} \right\} \end{aligned} \quad (15)$$

(iii) Equations Used for Analysis: Part B Plus Part D—The equations used for the analysis of the $(d(CG)_n)_2$ data are obtained by substituting eqs 6, 7, 11, 12, and 15 into eq 2 giving

$$Y_c(N) = B_c k \left[\frac{(m5-1)}{N} + \frac{1}{2} \right] + D_c k \frac{\left\{ \text{erf} \left[\frac{(N-d)}{w} \right] + 1 \right\}}{2} \times \frac{\{ \log[\cosh(\alpha(N-1)) \text{sech}(\alpha)] + m5 \log[\cosh(\alpha) \cosh(\alpha N) \text{sech}(\alpha(N-1))] \}}{\alpha^2(m3+m5-2+N)N} \quad (16a)$$

$$Y_g(N) = B_g k \left[\frac{(m3-1)}{N} + \frac{1}{2} \right] + D_g k \frac{\left\{ \text{erf} \left[\frac{(N-d)}{w} \right] + 1 \right\}}{2} \times \frac{\{ \log[\cosh(\alpha(N-1)) \text{sech}(\alpha)] + m3 \log[\cosh(\alpha) \cosh(\alpha N) \text{sech}(\alpha(N-1))] \}}{\alpha^2(m3+m5-2+N)N} \quad (16b)$$

$$R(N) = \frac{Y_g(N)}{Y_c(N)} \quad (16c)$$

The third equation, that for ratio R , is of course not an independent equation. It is used in the analysis as means of adding weight to the ratio R because R is less susceptible to systematic errors than Y_g or Y_c .

To describe the $(d(GC)_n)_2$ data, a set of three closely related, but different, equations were derived by interchanging $m5$ with $m3$ in eqs 16a and 16b. These new equations describe the fact that Gua and Cyt are at opposite ends. These six equations, sharing the same nine parameters, were used to fit the fbr data shown in Figure 2.

Analysis

We first attempted to fit the $(d(CG)_n)_2$ data with Part B of our formulation, eqs 6 and 7. The nonlinear least-squares fit was against three data sets: Gua yield, Cyt yield, and their ratio, R_i . These were simultaneously fit by the three equations with data weighted by $1/y^2$ ($y = y$ axis value). The hyperbolic curves derived from the fit are shown by dashes in Figure 2. The resulting four parameters, their standard deviations, 95% confidence limits, and the constraints applied are shown in Table 3 under the heading “Part B alone”. The goodness-of-fit (R^2 values) are shown near the bottom of Table 3 in the row labeled “Part B alone”. The values of $G(\text{Gua})$ were easily fit, $R^2 = 0.95$, whereas those of $G(\text{Cyt})$ were poorly fit, $R^2 = 0.16$. As a consequence, the Part B equations do a poor job of explaining the dependency of the ratio, $G(\text{Gua})/G(\text{Cyt})$, on N .

The Part B plus Part D equations, eqs 16a–16c, were applied in a similar fashion. For $(d(CG)_n)_2$, the yield of Gua, Cyt, and their ratio R were fit using these equations to simulate $Y_g(N)$, $Y_c(N)$, and $R(N)$, respectively. Included in this nonlinear least-squares fit were the data for $d(GC)_n)_2$ using three separate equations obtained by interchanging $m5$ and $m3$, as described above, and also using *M. luteus* for a data point at $N = 200$. These data were fit simultaneously with all nine parameters shared between the six equations; the data points were weighted by $1/y^2$. Constraints were set to keep parameters in physically realistic fitting space. The fit, which converged without hitting any of the constraints, is shown in Figure 2. The solid curves fit the

three sets of data points for $(d(CG)_n)_2$ and the dotted curves fit the data points for $(d(GC)_n)_2$. The nine parameters generating these fits are given in Table 2 along with their standard error, 95% confidence limits, and constraints employed. For $(d(CG)_n)_2$, the fit to $G(\text{Gua})$, $G(\text{Cyt})$, and $G(\text{Gua})/G(\text{Cyt})$ is relatively good, with R^2 values of 0.94, 0.85, and 0.91, respectively. For $(d(GC)_n)_2$, the R^2 values are negative because these three points would be better fit by a horizontal straight line. Noteworthy here, however, is the models ability to fit the $(d(GC)_n)_2$ with a resulting global R^2 value of 0.86. If the data points are not weighted by $1/y^2$, all of the R^2 values improve substantially, reflecting the fact that the values of $G(\text{Gua})$ are easier to fit and, being larger, contribute more to R^2 .

There are of course significant difficulties presented by the fact that the Part B plus Part D equations are over parametrized relative to the data sets being fit. As one might expect from the equations, there is large covariance between m_5 , B_{ck} , and D_{ck} and between m_3 , B_{gk} , and D_{gk} . The wide 95% confidence limits reflect this covariance and lack of sufficient constraints. It is important to note, however, that the parameter values are physically reasonable. Other mathematical models were tested that fit the data but none of these resulted in physically realistic parameters. It proved difficult to find a model that fit the $G(\text{Cyt})$ values and the ratios of $G(\text{Gua})/G(\text{Cyt})$, while simultaneously fitting data from *both* $(d(CG)_n)_2$ and $(d(GC)_n)_2$.

Discussion

The striking features of the data presented in Table 1 are the relatively flat response of the ratio R at small N , the corresponding dip in Cyt yield, and the drop in R when the sequence is reversed from $(d(CG)_n)_2$ to $(d(GC)_n)_2$. These features argue against using a simple hyperbolic function to describe the end effect and point toward a mechanism that engages the redox properties of the bases themselves, a mechanism such as that proposed above. Before discussing this mechanism, two alternative mechanisms are briefly considered.

Low energy electrons (LEE) are known to cause strand breaks,²⁵ presumably by dissociative electron attachment. Also, LEE induced sb are likely to be accompanied by free base release.²⁶ In addition, the action of LEE has been shown to be sensitive to base context in ss-DNA.²⁷ There is a good possibility, therefore, that LEE induced damage would show differences between the ends and interior of an oligomer. It is difficult, however, to see why an end-effect due to LEE would not follow a simple hyperbolic dependency. Further, to date, there is no evidence that indicates the yields of LEE induced strand breaks are large enough to account for the observed variation in yields of fbr, on the order of 70 nmol/J.

A second possibility is that the guanine radical cation, created by ionizing radiation in an electronically excited state, deprotonates preferentially at the strand ends. Adhikary et al. have shown that the photoexcited state of the guanine radical cation transfers the hole to its deoxyribose moiety resulting in hydrogen abstraction radicals at C1', C3', or C5'.^{28,29} Formation of these radicals is quenched at C3' or C5' by phosphorylation at the given carbon. Therefore, a Gua at either the C5' or C3' end would sensitize its own deoxyribose, as we observed for CG oligomers. Because this mechanism requires an electronically excited state, it can be argued further that the length of the base stack might influence the end effect so that the yield would drop more rapidly once N exceeds some critical number. Whereas this possibility cannot be dismissed, the yield of the obligatory-excited-guanine-radical cation is unknown for ionizing radiation. Also, by this explanation, it is difficult to reconcile the changes in Cyt release as a function of oligomer length.

By either of these alternative mechanisms, it is hard to explain the findings reported in Table 2. A sizable fraction of fbr yields cannot be accounted for by the yield of radicals trapped on the DNA backbone. As pointed out previously,^{8,15} a nonradical intermediate is produced at

relatively high yields (50–100 nmol/J). We have suggested, therefore, that deoxyribose carbocations formed by double oxidation play a prominent role, as shown in Schemes 2 and 3.

Two one-electron oxidations of the same deoxyribose requires that a second hole be formed in, or transferred to, an oligomer containing a deoxyribose hole. The probability of this occurring is related to the size of an ionization spur and the number of radicals therein. La Verne and Pimblott computed the radius of an average spur of 62.5 eV in water.³⁰ On the basis of a Gaussian distribution, they calculated that 60% of the ~6 primary radicals per spur are within a 4.46 nm radius and 90% within an 8.92 nm radius. Dividing these two values by 0.36 nm/bp (base pair distance in Z-form DNA) gives these two radii in dimensions of base pairs, ~12 and 25 bp, respectively. Our model treats the probability of finding two holes on the same oligomer using the erf function, with a center at distance d and width w . The best-fit values to the data (Figure 2 and Table 3) gave $d = 12.9$ bp and $w = 3.9$ bp. These values predict that the probability of two ionizations in the vicinity of a single oligomer should become discernible at about $((12.9 - 2) \times 3.9)$ bp ≈ 5 bp and should reach a plateau at about $((12.9 + 2) \times 3.9)$ bp ≈ 21 bp. This 5–21 bp range of response corresponds well with the calculated parameter that corresponds to spur dimensions of 12–25 bp.

Once two holes are formed on the same oligomer, the distance of hole transfer through the base stack governs the probability of a hole–hole encounter. This distance depends on the relative rates of trapping and transfer, described by the parameter α . Whereas values of α are unknown in solid state DNA, a value of 0.34 was calculated²⁴ from data on holes photochemically generated in DNA in dilute aqueous solution.³¹ The best fit value, $\alpha = 0.012$ in Table 2, is an order of magnitude smaller; this is reasonable given the expectation that the trapping rate should be significantly slower in the solid state.

The end effect, by definition, occurs only if m_5 and m_3 are greater than 1.0. A rough gauge of the magnitude of the end effect can be obtained from the reported yields of sb products in crystalline $d(\text{CGCG})_2$ and $d(\text{CGCGCG})_2$ irradiated at room temperature.⁹ In this work, the yield of shorter oligomers with 3'- or 5' phosphorylated end groups were measured using ion exchange HPLC. Cleavage of each phospho–ester bond was directly related to either one or two of these products depending on whether the damaged site was at a terminal or interior deoxyribose. From their data, one calculates $m_5 = 2.7$ and 1.9 and $m_3 = 1.9$ and 1.2 for $d(\text{CGCG})_2$ and $d(\text{CGCGCG})_2$, respectively. Comparing these with the best-fit values in Table 2, $m_5 = 5.3$ and $m_3 = 8.6$ appear too high. Given the large covariance between m_5 , B_gk , D_gk , and between m_3 , B_ck , and D_ck , it is not difficult to constrain m_3 and m_5 to values below 3 and still obtain good fits to the data. But such fitting exercises add little. At this stage, additional data is needed to provide tighter constraints on some of the parameters. Efforts are underway to do the later; potentially, m_3 and m_5 can be determined directly by measuring the release of modified bases synthetically incorporated at specific oligomer positions.

Conclusions

There is a substantial end effect when free base release is generated in DNA oligomers by the direct absorption of ionizing radiation, where oligomers are of the form $(d(\text{CG})_n)_2$ and $(d(\text{GC})_n)_2$. For $(d(\text{CG})_n)_2$, the ratio of the Gua yield to Cyt yield, R , is relatively constant at 2.4 ± 2.5 for $n = 2, 3$, and 4 ; it decreases to 1.2 as n increases from 5 to 10 ; and it plateaus at 1.0 ± 0.1 at large n . If Gua is moved to the 5' end, as in going from $d(\text{CG})_5$ to $d(\text{GC})_5$ for example, R drops from 1.9 ± 0.1 to 1.1 ± 0.1 . These effects are poorly described if the chemistry at the oligomer ends is assumed to be independent of the remainder of the oligomer. A model incorporating charge transfer through the base stack can be used to explain these effects.

The yield of free base release is substantially greater than the yield of trapped-deoxyribose radicals. Therefore, a large fraction of free base release stems from nonradical damage. It is proposed here that the nonradical damage consists of deoxyribose carbocations produced by two one-electron oxidations. This reaction pathway is enhanced by charge transfer through the base stack and is, thereby, a consistent way of explaining both the absence of trapped radical intermediates and the unusual trend in the release of Cyt relative to Gua as the oligomer length is increased.

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Figure 1. Free base release in ((CG)₅)₂ films at a hydration level of 2.5 waters per nucleotide: Cyt (●), Gua (○), and Cyt + Gua (▲). Straight line fits to points over the entire dose range are dashed lines and the fit to dose points <60 kGy are solid lines.

Figure 2.

Cyt and Gau release in (CG)_n and (GC)_n. The data point representing large N was arbitrarily set at $N = 200$ and these values of $G(\text{Gua})$ and $G(\text{Cyt})$ were calculated from the *M. luteus* data by extrapolating the yields measured for the actual GC content of 72% to a content of 100% (Table 1). The nonlinear-least-squares fit is shown as dashed lines for the one-part model. For the two-part model, the fit is shown as solid lines for the (CG)_n data and as dotted lines for the (GC)_n data.

Figure 3. Q-band EPR spectra of ((CG)₅)₂ films X-irradiated and observed at 4 K. The small vertical bar marks $g = 2.002$. At low dose, 9 kGy, the signal is due primarily to base-centered radicals. At high dose, 525 kGy, the broad signal due to deoxyribose radicals is more intense than that of the base-centered radicals. A signal due to radicals in the quartz sample tube also grows in at high dose.

Figure 4.

Free-radical dose response for $((CG)_5)_2$ films, at Γ 2.5, X-irradiated, and measured at 4 K. The signal due to quartz becomes apparent above 400 kGy and it was subtracted out to track the signal due to DNA alone, shown as solid circles. The inset shows the linearity of response at low dose. The solid curve is the least-squares fit to $C = (G_b/k_b)[1 - \exp(-k_b D)] + (G_d/k_d)[1 - \exp(-k_d D)]$, where C is radical concentration, G_b and G_d are the respective yields of base and deoxyribose radicals, k_b and k_d are the respective destruction cross sections for the base and deoxyribose radicals, and D is the dose. The values of $G_{dRib}(fr) = G_d$ and $G\Sigma(fr) = G_b + G_d$ are given in Table 2.

SCHEME 1.
Reaction Pathways Initiated by Direct Ionization of the Deoxyribose Moiety of DNA

SCHEME 2.

Proposed Mechanism by which the 3'-End Sensitizes Deoxyribose Damage in Oligodeoxynucleotides by the Direct Effect of Ionizing Radiation

SCHEME 3.

Diffusion-Dependent Double Oxidation of the Deoxyribose Requires Two Oxidation Events on the Oligomer: A Carbon-Centered Radical Trapped on Deoxyribose Due to Deprotonation of a Radical Cation and a Radical Cation Formed on the Base Stack that Diffuses through the Base Stack^a

^aIllustrated here is diffusion-dependent double oxidation resulting in carbocation formation at the 3'-end of DNA decamer.

TABLE 1

Chemical Yields of Free Base Release in GC Rich DNA Films Hydrated to 2.5 Mol Water/Mol Nucleotide; Values Based on *N* Experiments

dsDNA	<i>G</i> (Cyt) nmol/J	<i>G</i> (Gua) nmol/J	<i>G</i> (Thy) nmol/J	<i>G</i> (Ade) nmol/J	<i>R</i> = <i>G</i> (Gua)/ <i>G</i> (Cyt)	<i>G</i> (fbr) nmol/J	<i>N</i>
d((CG) ₂) ₂	53 ± 2	127 ± 12			2.39 ± 0.24	177 ± 9	2
d((CG) ₃) ₂	37 ± 2	85 ± 6			2.35 ± 0.21	124 ± 8	3
d((CG) ₄) ₂	32 ± 1	80 ± 7			2.48 ± 0.24	113 ± 8	2
d((CG) ₅) ₂	35 ± 2	67 ± 3			1.89 ± 0.13	102 ± 5	3
d((CG) ₆) ₂	38 ± 2	56 ± 3			1.45 ± 0.12	94 ± 5	2
d((CG) ₇) ₂	41 ± 2	55 ± 2			1.35 ± 0.06	96 ± 3	2
d((CG) ₁₀) ₂	49 ± 5	56 ± 5			1.14 ± 0.11	104 ± 9	2
d((GC) ₃) ₂	42 ± 7	41 ± 4			0.99 ± 0.11	83 ± 10	2
d((GC) ₆) ₂	46 ± 3	45 ± 4			0.98 ± 0.10	91 ± 7	2
d(GCGGCCCCGC) ₂	52 ± 6	57 ± 7			1.10 ± 0.15	109 ± 13	2
d(GCACGCGTGC) ₂	40 ± 5	41 ± 6	4 ± 2	8 ± 2	1.03 ± 0.20	93 ± 15	2
<i>M. luteus</i> (72% GC)	25 ± 1	25 ± 2	9 ± 1	8 ± 1	1.00 ± 0.10	65 ± 4	3
- projected to 100% GC -							
d(GCACGCGTGC) ₂	50 ± 6	51 ± 8			1.10 ± 0.15	101 ± 10	
<i>M. luteus</i> (72% GC)	34 ± 2	35 ± 3			1.00 ± 0.10	69 ± 4	

TABLE 2

Yield of Total Free Base Release, $G(\text{fbr})$, Compared with Yield of Total Radicals Trapped on DNA, $G\Sigma(\text{fr})$, and Yield of Trapped Deoxyribose Radicals, $G_{\text{dRib}}(\text{fr})$, in DNA Films Irradiated and Observed at 4 K^a

dsDNA	$G(\text{fbr})G\Sigma(\text{fr})$		$G_{\text{dRib}}(\text{fr})$	10–19% of $G\Sigma(\text{fr})$	$G(\text{fbr})-G_{\text{dRib}}(\text{fr})$
	measured nmol/J	measured nmol/J	measured nmol/J	estimated nmol/J	nmol/J
$d((\text{CG})_2)_2$	$180 \pm 9355 \pm 5$			36–67	145–113
$d((\text{CG})_3)_2$	$124 \pm 8450 \pm 5$			45–86	79–39
$d((\text{CG})_4)_2$	$113 \pm 8442 \pm 10$			44–84	69–29
$d((\text{CG})_5)_2$	$102 \pm 5295 \pm 33$		32 ± 1		70 ± 5
<i>M. luteus</i>	$63 \pm 5245 \pm 3$			25–47	39–16

^aThe free-radical yields for $d((\text{CG})_5)_2$ were determined by the curve fit shown in Figure 4. In the other four sequences, the values of $G\Sigma(\text{fr})$ were measured by a linear fit to dose points below 6 kGy (data not shown).

TABLE 3

Nonlinear Least-Square Fit of Free Base Release Yields in a Series of (CG)_n and (GC)_n Oligomers and Using *M. luteus* DNA (72% GC) to Represent the Data Point at $N = 200^a$

	values of fitting parameter			constraints
	best-fit	std. err.	95% confidence	
m3	6.4	0.7	5.1 to 7.7	m3 > 1.000 and shared
m5	1.5	0.2	1.0 to 1.9	m5 > 1.000 and shared
B_{gk}^k (nmol/l)	65	4	57 to 74	0 < B_{gk}^k < 500.0 and shared
B_{ck}^k (nmol/l)	71	3	64 to 78	0 < B_{ck}^k < 500.0 and shared
α	0.013	0.003	0.007 to 0.018	$a > 0.002$ and shared
d (bp)	12.7	0.6	11.6 to 13.9	$d > 2.0$ and shared
w (bp)	3.8	1.0	1.8 to 5.9	$w > 1.0$ and shared
m3	7.6	1.0	5.6 to 9.6	m3 > 1.0 and shared
m5	4.6	0.8	3.0 to 6.2	m5 > 1.0 and shared
B_{gk}^k (nmol/l)	55	6	43 to 66	0 < B_{gk}^k < 200.0 and shared
D_{gk}^k (nmol/l)	17	8	1 to 32	0 < D_{gk}^k < 200.0 and shared
B_{ck}^k (nmol/l)	34	5	25 to 44	0 < B_{ck}^k < 200.0 and shared
D_{ck}^k (nmol/l)	56	8	41 to 72	0 < D_{ck}^k < 200.0 and shared
G(Cyt)	G(Cyt)	Goodness-of-Fit, R^2 (unweighted) G(Gua)	G(Gua)/G(Cyt)	global
((CG) _n) ₂ data	0.16	0.95	0.76	
((CG) _n) ₂ data	0.85	0.94	0.91	0.86
((GC) _n) ₂ data	0.29	-0.60	-0.48	

^aThe data and the fit to the Part B equations, eqs 6 and 7, and the Part D plus Part B equations, eq 16, are shown in Figure 2.