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Reductive Damage in Directly Ionized DNA: Saturation of the C5=C6 Bond of Cytosine in d(CGCG)₂ Crystals

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Abstract

Electron paramagnetic resonance (EPR) was used to study an oligodeoxynucleotide duplex of d(CGCG)₂ that is known to crystallize in Z-form. After X irradiation at 4 K, EPR data were collected on single crystals and polycrystalline samples as a function of annealing temperature and dose. A radical produced by the net gain of a hydrogen atom at C6 and a proton at N3, Cyt(C6+H, N3+H⁺)[•], is identified. This radical had not been positively identified in polymeric DNA previously. The Cyt(C6+H, N3+H⁺)[•] makes up about 4% of the total radical population at 4 K, increasing to about 10–15% after the DNA is annealed to 240 K. There appears to be neither an increase nor a decrease in the absolute concentration of Cyt(C6+H, N3+H⁺)[•] upon annealing from 4 K to 240 K. Additionally, the presence of another radical, one due to the net gain of hydrogen at C5 of cytosine, the Cyt(C5+H)[•], is implicated. Together, these two radicals appear to account for 60–80% of the reduced species in DNA that has been irradiated at 4 K and annealed to 240 K.

INTRODUCTION

One of the primary methods used for identification of free radical intermediates in irradiated DNA and DNA components has been EPR. Studies of powders, films, frozen solutions, and single crystals of components have identified a great number of radical species and determined many of their EPR spectral characteristics. Single-crystal studies yield a wealth of detailed information because spectral resolution is generally good and ENDOR (electron nuclear double resonance) is often applicable. Amorphous polymeric DNA samples, such as powders and frozen solutions, yield relatively broad poorly resolved spectra that require spectral deconvolution and/or component analysis to identify specific radical species (1-4). To a large degree, deconvolution techniques rely heavily on the information garnered from single crystals and powder spectra of components to build a basis set of likely spectral components (often referred to as benchmark spectra). While this approach has made it possible to identify the major species produced in amorphous DNA, it is very difficult to identify minor species. This limitation comes from factors such as uncertainties in the benchmark spectra, lack of resolution in the powder spectra, and sample heterogeneity. Single crystals of low-molecular-weight DNA solve the problem of heterogeneity and to some degree enhance spectral resolution (5-7), thereby improving the chances of identifying radicals that are present as minor components.

Work on high-molecular-weight DNA irradiated at temperatures at or below 77 K has shown that the hole is trapped primarily by guanine, and the electron is trapped primarily by cytosine, and to a lesser extent on thymine (2,8-15). It is possible that adenine is also a minor electron

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trapping site (16). The pristine radical ions are believed to undergo reversible proton transfer, giving neutral radicals and stabilizing the trapping site; e.g., the cytosine radical anion protonates at N3, giving $\text{Cyt}(\text{N3}+\text{H})^\bullet$, and the guanine radical cation deprotonates, giving $\text{Gua}(\text{N1}-\text{H})^\bullet$ (17-20).

In the work presented here, we apply EPR to single crystals of $\text{d}(\text{CGCG})_2$ X-irradiated at 4 K. An important advantage of this system is that the tetramers stack one on top of the other, forming a continuous Z-DNA helix that is distinguishable from high-molecular-weight DNA only because the sugar-phosphate backbone is interrupted by a missing phosphate every 4 base pairs (21). The base stacks are not only continuous; they also run in straight columns, and the columns are parallel to one another. Thus all of the bases lie in parallel planes. This improves spectral resolution significantly, making it possible to identify one minor radical product with relatively high certainty and to make a sound proposal for the identity of another radical product. Both of these radicals are formed by reduction of the $\text{C5}=\text{C6}$ bond of cytosine.

MATERIALS AND METHODS

Crystals of $\text{d}(\text{CGCG})_2$ were grown following published procedures (21) from solutions containing the oligodeoxynucleotide, MgCl_2 and sodium cacodylate against a reservoir containing isopropyl alcohol. $\text{d}(\text{CGCG})_2$ may be grown into large rods ($\sim 2 \text{ mm} \times 0.3 \text{ mm} \times 0.3 \text{ mm}$) with a distinctive morphology that allows relatively accurate orientation of the crystal in the EPR cavity with respect to the external magnetic field, $\mathbf{B}_0$. The hydration state of the crystal has been estimated previously as 7.1 molecules of water per nucleotide (22), and the free radical yield at 4 K was found to be $0.65 \pm 0.15 \mu\text{mol/J}$ (6).

Single crystals were mounted on the ends of open 1.0-mm-diameter quartz capillaries (purchased from the Charles Supper Company) using a small amount of rubber cement. The thin-walled (0.1 mm) quartz tubes were chosen because the EPR signal induced by irradiation of the quartz is weak relative to that of the sample, minimizing the inevitable background due to the sample holder. The $\text{d}(\text{CGCG})_2$ crystals were mounted with the crystal's c axis (the crystal's long axis, corresponding to the DNA helix axis) perpendicular to the axis of the quartz tube. The variation from the desired orientation was estimated at $\pm 7^\circ$. Polycrystalline samples were placed in sealed quartz capillary tubes.

The crystals were irradiated at 4 K with a dose of at least 20 kGy. The dose rate of 26 kGy/h was provided by an OEG-76 tungsten filament X-ray tube maintained at a current of 20 mA and 70 keV. The samples were returned to the EPR cavity and allowed to equilibrate for several minutes before spectra were recorded with a Q-band Varian E-12 spectrometer (23).

Rotations of the mounted single crystals were monitored by a goniometer attached to the top of the sample rod. The crystal was positioned so as to place the c axis parallel to $\mathbf{B}_0$ near the rotation angle of 90° . The relative accuracy between the set of rotation angles was $\pm 2.5^\circ$. Where needed, g values were measured against the position of the hydrogen atom spectrum, which is produced by irradiation of the quartz sample holder.

Anneals were accomplished by raising the sample 40 cm above the cavity to the heater position, where the temperature was maintained to $\pm 3\%$ by a DRC-82 temperature controller from Lake Shore Cryotronics. The samples were normally kept at the annealing temperature for 10 min. The samples were then lowered back into the cavity and allowed to return to 4 K before spectra were again recorded.

Computational Procedures

The geometries, energetics and hyperfine properties of the cytosine C5 and C6 H-addition radicals were investigated by computational methods. All systems were optimized using the hybrid Hartree-Fock Density Functional Theory functional B3LYP (24-26), in conjunction with the 6-31G(d, p) basis set of Pople and coworkers (27). Frequency calculations were performed at the same level of theory to ensure that the systems represent true minima on the potential energy surfaces.

Single point calculations were performed on the optimized geometries, at the B3LYP/6-311G (2df,p) level, to obtain accurate energies and spin properties. All calculations were performed using the Gaussian 98 program package (28).

The combination of methods and basis sets employed in the present work has been used very successfully for determination of geometric structures, relative energies of different conformers, and radical hyperfine coupling constants (29-32). Overviews of the equations used for evaluating the different components of the diagonalized hyperfine interaction tensor within the DFT framework, and their performance, have been presented by Malkin *et al.* (33) and by Barone (34).

For calculations on the energetics of proton transfer between base pairs, procedures adopted by Sevilla and coworkers (35) were employed. The authors discuss the difference between non-relaxed and relaxed proton transfer. For the non-relaxed case, the proton on N1 of guanine is moved to the N3 of cytosine without optimization of the nuclear coordinates. The relaxed (or adiabatic) calculations involve full geometry optimizations of the base pairs performed at fixed N-H distances. In the present study, optimizations were performed with starting geometries obtained previously from studies of the cytosine anion, the N3 protonated cytosine, and the C6 H-addition radical. This is not quite the same as the adiabatic case, but it was shown to greatly speed up convergence.

RESULTS AND DISCUSSION

We first provide evidence that the $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ is trapped in crystalline $\text{d}(\text{CGCG})_2$ after X irradiation at 4 K (see Fig. 1 for the structure of this radical and other radicals discussed in the text). Its concentration remains relatively constant upon annealing to 240 K. Because annealing reduces the total radical concentration by about 80%, the fraction of the total radicals due to $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ increases from about 4% at 4 K to 10–15% at 240 K. Assuming that half the total radicals are formed by reduction and half by oxidation, there should be one or more other radicals produced by reduction that account for the “missing” 40% at 240 K. We propose that the $\text{Cyt}(\text{C5+H})^\bullet$ is likely to account for a large portion of this missing fraction.

A set of EPR spectra recorded for a $\text{d}(\text{CGCG})_2$ single crystal at 4 K after irradiation at 4 K is shown in Fig. 2. Rotation is about an axis that is perpendicular to the long axis of the crystal's rod-like habit. Since the rod axis is parallel to the DNA helix axis, the magnetic field ($\mathbf{B}_0$) can be aligned parallel to all the base planes; this occurs between the rotation angles of 0° and 15°. As the crystal is rotated 90°, to an angle of 90°–105°, $\mathbf{B}_0$ is aligned parallel to the DNA helix axis; i.e., it is perpendicular to the base planes. Spectra taken in this plane are consistent with a free radical population that is dominated by one-electron reduced cytosine protonated at N3 ($\text{Cyt}(\text{N3+H})^\bullet$) (36) and one-electron oxidized guanine deprotonated at N1 [$\text{Gua}(\text{N1-H})^\bullet$] (37). The former gives a doublet spectrum while the latter gives a broad singlet when $\mathbf{B}_0$ lies in the base plane (0°–15° in Fig. 2). The spectra of both species are broadened by hyperfine coupling to the ring nitrogens when $\mathbf{B}_0$ is perpendicular to the base plane (90°–105°). The intense spectra of these two radicals make it difficult to uncover other radicals that are present in smaller amounts. Thermal annealing reveals the presence of at least two additional radical types.

Annealing to 240 K reduces the total radical concentration to about 20% of the concentration at 4 K (20). This is a consequence of combination reactions between liberated $\text{Gua}(\text{N1-H})^\bullet$ and $\text{Cyt}(\text{N3+H})^\bullet$, where liberation is believed to involve a reversible proton transfer back to guanine, forming the guanine radical cation which then may tunnel to nearby guanines (20, 38). The 240 K annealing reduces the concentration of these two radicals substantially. This can be seen from the plane of data, shown in Fig. 3, taken on the same crystal in the same orientation as in Fig. 2. Spectra of other radical species are now revealed.

It is possible to identify one of the radical species with a relatively high level of confidence, using just the outer features of spectra taken after annealing to 240 K. Pivotal to the radical assignment is the spectral width, which in this plane of data varies from 11.8 mT to about 12 mT. There is only one radical, known to be formed in nucleic acid constituents, that has a corresponding spectral width, the $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ [see (39) and Table 1]. The broadest spectrum due to a sugar radical in Z-DNA would not be sufficient to account for these features (Close, unpublished results). The spectral width of the phosphite radical (60–70 mT) (40) is too large. The radical formed by the net gain of hydrogen at C8 of guanine gives a triplet spectrum that is about 7.2 mT wide (41–43). The spectrum due to the radical formed by the net gain of hydrogen at C5 of cytosine, the $\text{Cyt}(\text{C5+H})^\bullet$, has a width ≤ 9.3 mT (44–47).

The measured width of the spectra for $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$, in various crystalline systems, is 11.5–12.0 mT (44,45,47–49). The isotropic hyperfine coupling to the α -hydrogen at C5 is 1.67–1.78 mT, depending on the derivative crystal (44,45,48,49), and the two β -hydrogen couplings at C6 sum to 9.5–10.2 mT (39,44,47). In Fig. 3, the splitting between the outermost line (on the high- and low-field sides) and the next line in is about 1.7 mT. This splitting displays some anisotropy. Given the crystal space group of P6_5 and an effective asymmetric unit of 6 base pairs (21), there are generally six magnetically distinct trapping sites for $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ (or any base radical). When $\mathbf{B}_0$ is perpendicular to the base planes (100° – 110° in Fig. 3), all of these sites are magnetically equivalent. Thus, at this orientation, the intermediate principal value of the α -hydrogen coupling tensor should be observed. This is expected to be 1.6–1.8 mT (44,45,48–50), and 1.7 mT is observed. When $\mathbf{B}_0$ is parallel to the base planes, the six magnetically distinct sites will give couplings that vary between the maximum principal value of 2.3–2.8 mT and the minimum of 0.6–0.9 mT (46,47,50,51). This is consistent with broadening of these features as the crystal is rotated from $\mathbf{B}_0 \perp$ base planes to $\parallel$ base planes (i.e., from 90° to 0°). The evidence is rather strong, therefore, that the outermost lines are due to a radical formed by the net gain of hydrogen at C6 of cytosine.

Previous work indicates that protonation at N3, giving the $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ cation instead of the neutral $\text{Cyt}(\text{C6+H})^\bullet$, is necessary to explain the large β -hydrogen couplings [see ref. (39)]. The results of the Density Functional Theory (DFT) calculations performed on these radicals formed in 1-methylcytosine are given in Table 2. In spite of the fact that planar rings were assumed, there is excellent agreement between observed and calculated hyperfine couplings. Adding a proton to N3 of the C6-H adduct is seen to increase the spectral width by 1.1 mT. Thus the evidence is rather compelling that the $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ is formed in d(CGCG)₂, and this means that cytosine's conjugate base, guanine, donates a proton in the course of this radical's formation.

The $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ cation has been detected in irradiated 5'dCMP (46) and dGMP:dCMP, but not in polyG:polyC or poly[dGdC]:poly[dGdC] (12). It has been tentatively identified in DNA (52).

The EPR powder spectrum of $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ was simulated using parameters described in the paper by Hole *et al.* (47) and the spectral simulation software developed in our laboratory (53). The experimental spectrum of an annealed polycrystalline d(CGCG)₂ sample is shown

in Fig. 4a, and the simulated spectrum of $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ is shown in Fig. 4b. From integration of the two spectra, it is estimated that 10–15% of the $\text{d}(\text{CGCG})_2$ spectrum (after annealing to 240 K) is made up of $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ and about 4% at 4 K (no annealing).

As can be seen from Fig. 5, the $[\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}] : [\text{total radical}]$ ratio increases after annealing, while the absolute amount of $[\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}]$ does not change. Thus the $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ is formed at 4 K and the large population of $\text{Cyt}(\text{N3+H})^\bullet$ is not converted, at least to a measurable extent, into $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ upon annealing. The primary fate of the $\text{Cyt}(\text{N3+H})^\bullet$ is almost certainly annihilation by combination reactions with holes that are thermally liberated from $\text{G}(\text{N1-H})^\bullet$ trapping sites (20,46). These findings have important mechanistic implications and are discussed below.

Working under the assumption that for each radical produced by oxidation a radical is produced by reduction, we expect that 50% of the radical population should be formed by electron or hydrogen atom addition. Focusing attention on the radical population found after annealing to 240 K, only 10–15% of the radicals are of the type $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$. Thus these account for only 20–30% of the expected radicals produced by reduction. This prompted us to look for evidence of $\text{Cyt}(\text{C5+H})^\bullet / \text{Cyt}(\text{C5+H}, \text{N3+H}^+)^{+\bullet}$ (these two radicals are not distinguishable under our conditions). The powder spectrum of either of these species is expected to be a sextet with line intensity ratios of roughly 1:2:1:1:2:1 and a spectral width of ~9 mT (44–47). A simulated spectrum is shown in Fig. 4d. The spectrum in Fig. 4c was obtained by subtracting the simulated spectrum of $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ (Fig. 4b) from the experimental spectrum (Fig. 4a). As can be seen by comparing the difference spectrum, 4c, with that of 4d, the presence of the $\text{Cyt}(\text{C5+H})^\bullet / \text{Cyt}(\text{C5+H}, \text{N3+H}^+)^{+\bullet}$ appears quite likely. Assuming this to be the case, we fitted the outer lines of the simulated spectrum to the outer lines of the difference spectrum to obtain an estimate of this radical's concentration. The presumed $\text{Cyt}(\text{C5+H})^\bullet / \text{Cyt}(\text{C5+H}, \text{N3+H}^+)^{+\bullet}$ accounts for 20–25% of the total radical population. Based on reduced-to-oxidized stoichiometry of 1:1, it accounts for 40–50% of the radicals formed by reduction pathways. By this analysis, 60–80% of the reduced radicals are accounted for in $\text{d}(\text{CGCG})_2$ annealed to 240 K. Given the inherent limitations in using such simulated spectra and the possibility that the spectral width of selected sugar radicals approaches the 9-mT width of $\text{Cyt}(\text{C5+H})^\bullet$, the above analysis gives a reasonable accounting of radical species produced by reduction.

Consideration of the mechanisms underlying the formation of these radicals leads to the question of whether $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ is formed by the action of one or more than one radiation track. If it is one track, the radical concentration should increase linearly with dose. If two tracks are required, the increase in concentration should show a quadratic dependence on dose. Shown in Fig. 6 is the dose–response curve for the fraction of the total radicals assigned to $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$. It appears to follow the standard curve for total free radical production (6), which is linear from zero to 5 kGy, bending over between 5 kGy and 15 kGy, and flat above 15 kGy. Due to the large errors in measuring the amount of the $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ present, one cannot be very confident that the initial response is linear. However, there is fairly high certainty that above 10 kGy the curve is either flat or descending. Given that the sugar radical population continues to grow in at higher doses [see refs. (40,54,55)], one would expect that the ratio of base radical concentration to total radical concentration must decline at high dose. If a two-hit mechanism is required, then the concentration in the 10–40-kGy region should be increasing sharply. Since this is not the case, it provides good evidence that the $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ is produced by the interaction of a single track.

Here we briefly consider two mechanisms for the formation of $\text{Cyt}(\text{C6+H}, \text{N3+H}^+)^{+\bullet}$ by a single track. The first is homolytic cleavage of a sugar group CH bond, producing a hydrogen atom that subsequently adds to C6 and/or C5 of cytosine. The second is electron capture by cytosine followed by proton transfer from N1 of guanine to N3 of cytosine and capture of second proton

at C6 and/or C5. In the former case, hydrogen-atom addition to C6 must be followed by proton transfer from guanine to cytosine. In the latter case, the proton adding to C6/C5 must be generated by ionization of a nearby constituent; for example, deprotonation of a radical cation located on the sugar or phosphate moieties. While the hydrogen-atom addition mechanism requires only a single event [homolytic dissociation from an excited state or perhaps dissociative electron attachment (56-58)], the double protonation of an electron adduct requires spatially correlated events, i.e. an ionization cluster. The cluster would have to contain at least one hole and one trapped electron.

We prefer the clustered-events explanation for several reasons. One is that it does not seem likely that hydrogen addition at C6 will drive proton transfer [as it does when cytosine is one-electron reduced (17)]. To test this, calculations were performed on planar base pairs using the coordinates from Rosenberg *et al.* (59). In the reduction of GpC to produce the GC radical anion, it was noticed that there are non-planarities in the cytosine C4-NH₂, and that the cytosine and guanine bases moved closer together. Since it is not clear whether these two situations would actually occur in the d(CGCG)₂ crystals, the molecules were constrained to planar geometries, and the cytosine-to-guanine distance was kept constant. Preliminary calculations were performed using these constraints at the 6–31 G level. For electron addition to a C:G base pair, shown in Table 3, the results agree with those of Colson *et al.* (35); that is, proton transfer from N1 of guanine to N3 of the cytosine anion is energetically favorable (by 10 kJ/mol in our calculation and 20 kJ/mol in that by Colson *et al.* using the smaller 3–21 G basis set). For hydrogen addition to C6 of cytosine, we find that proton transfer is unfavorable by 125 kJ/mol.

Another reason for preferring the cluster-event explanation is that if hydrogen atoms are generated with a high enough yield to account quantitatively for the Cyt(C6+H,N3+H⁺)⁺⁺, then we would anticipate hydrogen adducts for the other three bases in DNA. Hydrogen atoms react with all four bases relatively indiscriminately: Using hydrogen-atom bombardment, Holroyd and Glass found the ratios of 49:23:~10:~20 for hydrogen-atom reactions with Gua +Ade:Thy:Cyt:sugar (60). There is no good evidence to date of hydrogen addition to guanine or adenine in solid-state DNA. In the case of d(CGCG)₂, the smallest ratio predicted for hydrogen-atom addition to Gua vs. Cyt is 49: 33, based on simply summing the above numbers for Thy and Cyt in the above ratios. At a minimum, therefore, one would predict 15–25% of the radicals in d(CGCG)₂ crystals, after annealing to 240 K, to be due to hydrogen addition to C8 of guanine. One would thereby expect the 7-mT-wide 1:2:1 triplet characteristic of hydrogen addition to C8 of guanine to be apparent, particularly at the 90° orientation in Fig. 3. Since it is not, this argues that pathways involving attack by thermalized hydrogens are relatively unlikely.

Future work will involve crystalline DNA that contains thymine and adenine bases. The goal of these studies will be to ascertain the presence of radicals produced by the net gain of hydrogen at the C5=C6 aromatic bond of both the pyrimidine bases, and to determine the capability of thymine to compete with cytosine as an electron radical trap.

CONCLUSION

We believe this is the first report in which the structure of base centered radicals is identified in crystalline oligonucleotide DNA. Although the DNA is in the Z-form, it is noteworthy that the base stacking is continuous, extending seamlessly through the crystal (up to several millimeters). The identified radical is centered on cytosine and is formed by the net gain of hydrogen at C6 and proton transfer to N3 [the Cyt(C6+H,N3+H⁺)⁺⁺]. It is created at 4 K (making up about 4% of the total radical population) and it remains stable to at least 240 K, where it makes up 10–15% of the radical population. Since thermal annealing does not produce Cyt(C6+H,N3+H⁺)⁺⁺ to a measurable extent, the population of stably trapped N3 protonated

cytosine radical anions, $\text{Cyt}(\text{N3}+\text{H}^+)^{\bullet}$, is not a significant source of precursor to $\text{Cyt}(\text{C6}+\text{H}, \text{N3}+\text{H}^+)^{+\bullet}$ formation.

Under the assumption that 50% of the radical population is derived through the reductive pathway, it is proposed that other related species should be present in comparable amounts. The radical formed by the net gain of hydrogen at C5 is found to be consistent with the data and fills this requirement, accounting for an estimated 20–30% of the total radical population after annealing to 240 K. Thus the net gain of hydrogen at the C5=C6 double bond can account for most if not all of the reductive radical damage stabilized after annealing $\text{d}(\text{CGCG})_2$ to 240 K.

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FIG. 1.
Structures and labels of the radicals discussed in the text.

FIG. 2.

Q-band EPR spectra of a single crystal of $d(CGCG)_2$ irradiated with a dose of 22 kGy at 4 K, and rotated about the a/b axis (an axis normal to c) in 15° increments. The crystal was mounted so that the c axis [corresponding to the long axis of the crystal and the helix axis of the $d(CGCG)_2$ duplex] is parallel to $\mathbf{B}_0$ at an angle of $\sim 97^\circ$. The base planes in the DNA crystal are oriented such that they are parallel to $\mathbf{B}_0$ at $\sim 7^\circ$. The scan width is 40 mT with the field centered on 1258 mT. The scans were produced using a 0.3-s time constant and 0.2 mT peak-to-peak modulation. Sweep time was 8 min, with a minimum of four sweeps averaged for each spectrum.

d(CGCG) Annealed to 240 K

FIG. 3. Q-band EPR spectra of the same crystal as in Fig. 2 but after annealing to 240 K for 10 min. The crystal orientation is as described in Fig. 2, but it was rotated in 10° increments.

FIG. 4.

(a) Spectrum of polycrystalline $d(\text{CGCG})_2$ after irradiation with a dose of approximately 60 kGy at 4 K followed by an annealing to 240 K. The scan is 20 mT wide. (b) Simulated spectrum of $\text{Cyt}(\text{C6}+\text{H}, \text{N3}+\text{H}^+)^{\bullet}$. The simulated spectrum assumed an anisotropic g tensor (2.0034, 2.0021, 2.0039) and slightly anisotropic hyperfine couplings from two β -hydrogens (5.1, 4.9, 4.7 and 5.0, 4.9, 4.7 mT), and coupling to one α -hydrogen of 2.5, 1.7, 0.8 mT [see (46)]. A 1.0-mT isotropic line width was used. The resulting spectrum obtained from subtraction of the simulated spectrum from that taken at 240 K is shown as (c). A simulated spectrum of $\text{C}(\text{C5}+\text{H})^{\bullet}$ is shown in (d) [the hyperfine couplings were taken from Hole *et al.* (46), and the g tensor is from Rustgi and Box (44)].

FIG. 5.

Fraction of the experimental spectrum of $d(\text{CGCG})_2$ made up of $\text{Cyt}(\text{C6+H,N3+H}^+)^{++}$ (diamonds) and $\text{Cyt}(\text{C5+H})^*$ (circles). The fraction was calculated by comparison of the simulated spectra to the annealed experimental spectra (solid symbols) and by comparison of the simulated spectra to the total initial radical population at 4 K (open symbols).

FIG. 6.

Fraction of experimental spectrum of $d(CGCG)_2$ that is made up of the $Cyt(C6+H,N3+H^+)^{+\bullet}$ species as determined from comparison of the experimental spectrum and the simulated spectrum as a function of dose. The line is the least-squares fit to the data, indicating that the concentration at high doses is either constant or decreasing.

TABLE 1

Relevant Characteristics Used for the Identification of the Radical Species Contained in the d(CGCG)₂ Oligonucleotide Crystals

Radical	Spectral width (mT)	α -H isotropic coupling (mT)	Reference(s)
Putative C(C6+H,N3+H ⁺) ⁺⁺	11.8–12.0	1.7	This work
C(C5+H) [•]	8.6–9.3	1.74–1.86	(44,47)
C(C6+H) [•]	11.5–12.0	1.67–1.78	(44,45,47–49)
G(G8+H) [•]	7.0–7.2		(41,43)
Phosphate	61.0–78.0		(40,61)
Sugar	≤8.5		(62)

Hyperfine Coupling (hfc) of the α - and β -Hydrogens in Carbon H-Adducts of 1-Methylcytosine Calculated by Density Functional Theory and Compared with EPR Measurements

TABLE 2

Radical type	iso α -hfc (mT)	iso β -H hfc (mT)	Spectral width (mT)	Energy (arbitrary units)	Reference(s)
Cyt(C6+H) [*]	-16.3	43.3	103.0	-434.94368	This work
Cyt(C6+H,N3+H ⁺) ^{**}	-16.3	49.0	114.2	-435.32712	This work
Observed C6 H-add	-17.8	47.8/51.3	116.9		(44)
Cyt(C5+H) [*]	-18.8	34.5	87.7	-434.94604	This work
Cyt(C5+H,N3+H ⁺) ^{**}	-19.8	33.7	87.2	-435.32447	This work
Observed C5 H-add	-17.4	30.8/45.2	93.4		(44)

TABLE 3

Comparison of Energies, Calculated at the 6-31 G Level, for the G:C Base Pair Reduced by either One Electron or by Hydrogen Addition to C6 of Cytosine

Radical	Energy
Gua:Cyt ^{-•} →	-1009.611482 arbitrary units
Gua(N1-H ⁺) ^{-•} :Cyt(N3+H ⁺) [•]	-1009.615214 arbitrary units ΔE = -10 kJ/mol
Gua:Cyt(C6+H) [•] →	-1010.174354 arbitrary units
Gua(N1-H ⁺) [•] :Cyt(C6+H,N3+H ⁺) ^{+•}	-1010.126821 arbitrary units ΔE = +125 kJ/mol

Note: In each case, the comparison is between all protons in their natural positions and the case where the guanine N1 proton is transferred to N3 of cytosine.