The probability of double-strand breaks in giant DNA decreases markedly as the DNA concentration increases.

Shunsuke F. Shimobayashi
Department of Physics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan

Takafumi Iwaki
Fukui Institute for Fundamental Chemistry, Kyoto University, Kyoto 606-8103, Japan

Toshiaki Mori
Radiation Research Center, Osaka Prefecture University, Sakai 599-8570, Japan

Kenichi Yoshikawa
†
Department of Physics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan and Faculty of Life and Medical Sciences, Doshisha University, Kyoto 610-0394, Japan

(Dated: May 1, 2014)

DNA double-strand breaks (DSBs) represent a serious source of damage for all living things and thus there have been many quantitative studies of DSBs both in vivo and in vitro [1–7]. Despite this fact, the processes that lead to their production have not yet been clearly understood, and there is no established theory that can account for the statistics of their production, in particular, the number of DSBs per base pair per unit Gy, here denoted by $P_1$, which is the most important parameter for evaluating the degree of risk posed by DSBs. Here, using the single-molecule observation method with giant DNA molecules (166 kbp), we evaluate the number of DSBs caused by $\gamma$-ray irradiation. We find that $P_1$ is nearly inversely proportional to the DNA concentration above a certain threshold DNA concentration. A simple model that accounts for the marked decrease of $P_1$ shows that it is necessary to consider the characteristics of giant DNA molecules as semiflexible polymers to interpret the intrinsic mechanism of DSBs.

As for DSBs caused by $\gamma$-rays in vitro experiments, its reported values scatter between $7.1 \times 10^{-9}$ and $2.1 \times 10^{-6}$ (expressed as the number of DSBs per base pair per unit Gy). The scattered data may be attributed to significantly varying DNA concentrations and the sizes of DNA. Although gel electrophoresis has been the most widely used method to enumerate DSBs, it requires a high DNA concentration (more than several tens of $\mu$M in base pair units) [1–7]. Contrastingly, the single-molecule observation method employed in the present study is applicable to systems with DNA concentrations as small as three orders of magnitude smaller than those used in gel electrophoresis [6]. In this article, we report the marked dependence of DSBs on the DNA concentration over a wide range of concentrations, indicating the significance for genome-sized DNA.

Figure I exemplifies the fluorescence microscopic images that we obtained of the DNA in solution on the glass substrate. We obtained the average length of molecules irradiated with a dose $I$, $L(I)$, for a range of $I$ and thereby evaluated the number of DSBs per base pair, denoted by $P_0$ (see the Methods section).
FIG. 2. Irradiation dose dependence of the number of DSBs per base pair. The number of DSBs per base pair, \( P_0 \), as a function of the irradiation dose from the Co\(^{60} \) source, \( I \). The data points for four different concentrations of samples are plotted.

FIG. 3. DNA base-pair concentration dependence of the number of DSBs per base pair per unit Gy. Log-log plot of the number of DSBs per base pair per Gy, \( P_1 \), as a function of the DNA base-pair concentration, \( C_{bp} \). It is seen that \( P_1 \) is roughly constant for small values of \( C_{bp} \) (compare with the solid line of zero slope) and inversely proportional to \( C_{bp} \) for large values. The blue dashed line represents a least-squares fit to the data using equation (5). The best fit is obtained with \( \frac{N_{rad}}{V_{tot}} = 1 \times 10^{-31} \mu M (100 \text{kbp} \cdot \text{Gy})^{-1} \) and \( \frac{V}{N_{bp}} = 1.38 \mu M^{-1} \).

Figure 2 displays the dependence of \( P_0 \) on \( I \). In the range of DNA concentrations from 0.015 to 300 \( \mu M \) in base pair units, \( P_0 \) for all samples showed linear dependences on \( I \). Thus, \( P_1 \) for a given \( C_{bp} \), the DNA base-pair concentration, is uniquely determined as the slope of the corresponding line representing \( P_0(I) \).

Figure 3 contains a log-log plot of \( P_1 \) as a function of \( C_{bp} \). Two different regimes are clearly seen. First, in the low concentration region, the quantity \( P_1 \) is nearly independent of \( C_{bp} \). Second, in the high region, \( P_1 \) decreases as a function of \( C_{bp} \), being approximately proportional to \( C_{bp}^{-1} \).

FIG. 4. Schematic illustration of the interaction between a reactive species and DNA molecules. We assume that a DSB is produced with a fixed probability \( q \), provided that at least one DNA molecule exists in the sphere of radius \( R_{rea} + R_g \).

The interaction of DNA molecules with ionizing radiation can be separated into two categories: "direct effects" (from energy deposited in the DNA and hydrated water molecules) and "indirect effects" (from free radicals produced by energy deposited in water molecules and other biomolecules located close to the DNA). In dilute solution, the ratio of the energy deposited in DNA and hydrated water molecules would be much smaller than...
that deposited in the other molecules of the solution. In addition, Ito et al. has shown that the contribution of indirect effects to DSBs at $C_{bp} \approx 100 \mu M$ is approximately two orders of magnitude larger than that of direct effects $^7$. Therefore it is reasonable to assume that indirect effects play a dominant role in the production of DSBs in our experiment.

Now we propose a simple model to account for our experimental data. In this model, it is assumed that each $\gamma$-ray photon deposits energy along the radiation track mainly through Compton scattering (see FIG.4 $^{12}$) and produces reactive species $^{13}$. Each reactive species has an effective diffusion length $R_{rea}$. According to a scaling argument, the radius of gyration $R_g$ can be regarded as defining the dimensions of a real DNA chain in a good solvent $^{14}$. Given that at least one DNA molecule exists in the sphere of radius $R_{rea} + R_g$, we assume that a DSB is produced with a fixed probability $q$ (see FIG.4). The sphere positioned at the center-of-mass of a reactive species means the gsphere of accessibility” for that reactive species: It is capable of attacking any DNA molecule located within in this sphere. Based on our framework, $P_1$ is given by

$$P_1 = \frac{N_{rea}q(1 - Q)}{N_{bp}N_{DNA}},$$

where $N_{rea}$ is the number of reactive species produced per unit Gy, $Q$ is the probability that no DNA molecule exists in a given sphere, $N_{bp}$ is the number of base pairs for a single DNA molecule, and $N_{DNA}$ is the number of DNA molecules in the sample of total volume $V_{tot}$. The quantity $Q$ is obtained by assuming that a single DNA molecule uniformly distributes in the region, that has the radius $R_{rea} + R_g$ and the volume $V$, in the sample of total volume $V_{tot}$ and that the positions of all DNA molecules are mutually independent variables. This yields the following:

$$Q = (1 - \frac{V}{V_{tot}})^{N_{DNA}}.$$  

Next, because $N_{DNA}$ is given in terms of $C_{bp}$ as

$$N_{DNA} = \frac{V_{tot}C_{bp}}{N_{bp}}$$

for $V \ll V_{tot}$, we obtain

$$Q \approx e^{-\frac{V}{V_{tot}}C_{bp}}.$$  

Then, combining equations (1), (3) and (4), we find

$$P_1 \approx \frac{N_{rea}q(1 - e^{-\frac{V}{V_{tot}}C_{bp}})}{V_{tot}C_{bp}}.$$  

We have fitted our data with equation (2) by treating $N_{rea}q/V_{tot}$ and $V/N_{bp}$ as fitting parameters (see FIG.3). The best fit is obtained with $N_{rea}q/V_{tot} = 1.31 \mu M (100kbp \cdot Gy)^{-1}$ and $V/N_{bp} = 1.38 (\mu M)^{-1}$. In addition, $V/N_{bp}$ is given in terms of $R_{rea}$ and $R_g$ as

$$\frac{V}{N_{bp}} = \frac{4}{3}\pi\left(R_{rea} + R_g\right)^3.$$  

Then, substituting the best-fit value for $V/N_{bp}$ and the values $N_{bp} = 166 \times 10^3$ and $R_g = 1.5 \mu m$ $^{13-16}$, we obtain $R_{rea} \approx 1 \mu m$ from equation (6). Finally, the effective lifetime of the reactive species, $\tau$, is estimated to be on the order of one millisecond from the relation $R_{rea}^2 \tau \approx D\eta$, where $D$ ($\approx 10^{-9} m^2/s$) is the diffusion coefficient $^{17}$. This value of $\tau$ is reasonable, because the reactive species exist for approximately a millisecond $^{18}$.

The functional form of equation (5) suggests that the threshold concentration $C_{bp}^*$ can be defined as the following relation:

$$C_{bp}^* \sim \frac{N_{bp}}{V}.$$  

The concentration $C_{bp}^*$ corresponds to the situation in which each region of volume $V$ contains one DNA molecule and each reactive species attacks just one DNA molecule, on average. Therefore, the dependence in the high concentration region can be understood from the conjecture that the number of reactive species that can possibly attack a DNA molecule is essentially independent of $C_{bp}$, and thus increasing $C_{bp}$ results in the reduction of such reactive species per DNA molecule.

Furthermore, we take into account the size of DNA in order to study its dependence on $C_{bp}^*$. From equations (6) and (7) and the relations $N_{bp} = \frac{Z}{\eta}$ and $R_g \approx \frac{2}{3}\lambda^2 Z^{3/5}$ $^{14-16}$, $C_{bp}^*$ is obtained as follows:

$$C_{bp}^* = \frac{Z}{\eta} \frac{1}{\frac{4}{3}\pi\left(R_{rea} + \frac{2}{3}\lambda^2 Z^{3/5}\right)^3},$$

where $\eta$ (=3.4 Å) is the distance between adjacent base pairs, $\lambda_k$ is the Kuhn length, and $Z$ is the total length of a single DNA molecule. The quantity $C_{bp}^*$ given by equation (8) is plotted as a function of $Z$ in the case that $R_{rea} = 1 \mu m$ and $\lambda_k = 0.1 \mu m$ in FIG.5. This figure represents a phase diagram of $C_{bp}^*$ and $Z$: In region A, $P_1$ is independent of $C_{bp}$, while in region B, $P_1$ is inversely proportional to $C_{bp}$.

We now consider the process in which a single DSB is induced by reactive species. From the relations $P_0 \propto I$ (see FIG.2) and $I \propto N_{pho}$ (the number of irradiating photons), we obtain the relation $P_0 \propto N_{pho}$. This assumes that DSB events produced by different photons are independent. Each gamma-photon produces clusters of reactive species along the radiation track $^{13,19}$. The diameters of these clusters are several nanometers, and they are widely separated in the case of radiation with low linear energy transfer $^{13,19}$. Furthermore, as shown in FIG.5...
After the irradiation, we added YO-YO-1 at a dose rate of 0.002-7 Gy/min. The fluorescent dye poly-L-lysine solution (Sigma-Aldrich, USA), and a Japan was cationically modified with a 0.05% (v/v) 2-mercaptoethanol (Nacalai Tesque, Japan) at 4% (v/v) substrate, a glass base dish of 35 mm diameter (Iwaki, Japan) was used to avoid additional fragmentation by mechanical stress. Fluorescence images of DNA molecules were observed using an Axiovert 135 TV (Carl Zeiss, Germany) fluorescence microscope equipped with a 100× oil-immersed objective lens. The lengths of the elongated DNA molecules were measured using cosmos image-analysis software (Library, Japan). We measured the contour lengths of approximately 100 molecules in each sample solution and thereby obtained L(I). Giant DNA molecules exceeding 100kb are occasionally inadvertently cut into fragments during the sample preparation, which includes pipetting and mixing. For this reason, the value of L(0) is slightly shorter than the natural length of T4 DNA, 57 μm. The quantity P0 (the number of DSBs per base pair) is calculated from the difference between the average molecular lengths of irradiated and non-irradiated samples that are otherwise identically prepared:

\[ P_0 = \frac{L(0) - L(I)}{L(0)} \cdot \eta. \]

Here, \( \eta \) (=3.4 Å) is the distance between adjacent base pairs.

**METHODS**

Experimental procedures and evaluation method. We used T4 phage DNA (166kbp, Nippon Gene) in a 20 mM phosphate buffer (pH 7.3) solvent. DNA was irradiated by γ-rays from a Co\(^{60}\) source at a dose rate of 0.0027 Gy/min. The fluorescent dye YO-YO-1 (Abs/Em 491/509 nm, Molecular Probes, USA) was used for visualization of the DNA chains. After the irradiation, we added YO-YO-1 at 1μM and 2-mercaptoethanol (Nacalai Tesque, Japan) at 4% (v/v) at the final concentration in order to avoid additional oxidative damage. To fix the DNA molecules on a glass substrate, a glass base dish of 35 mm diameter (Iwaki, Japan) was cationically modified with a 0.05% (v/v) poly-L-lysine solution (Sigma-Aldrich, USA), and a sample solution (~5 μl) was adsorbed onto the modified glass base dish. To elongate the DNA molecules, the droplet of sample solution was covered with a glass slip (18 mm×18 mm). We applied a weak shear to the DNA molecules by sliding this glass slip. This was done very gently to avoid additional fragmentations.
by γ-ray in DNA on the physical conditions of exposure: water content and temperature. *Int. J. Radiat. Biol.* **63**, 289-296 (1993).

[8] Boudaiffa, B., Cloutier, P., Hunting, D., Huels, M. A., & Sanche, L. Resonant Formation of DNA Strand Breaks by Low-Energy (3 to 20 eV) Electrons. *Science* **287**, 1658-1660 (2000).

[9] Martin, F., Burrow, P. D., Cai, Z., Hunting, D. & Sanche, L. DNA Strand Breaks Induced by 0-4 eV Electrons: The Role of Shape Resonances. *Phys. Rev. Lett.* **93**, 068101 (2004).

[10] Wang, C.-R., Nguyen, J., & Lu, Q.-B. Bond Breaks of Nucleotides by Dissociative Electron Transfer of Nonequilibrium Prehydrated Electrons: A New Molecular Mechanism for Reductive DNA Damage. *J. Am. Chem. Soc.* **131**, 11320-11322 (2009).

[11] Michael, B. D., & O’Neill, P. A Sting in the Tail of Electron Tracks. *Science* **287**, 1603-1604 (2000).

[12] Davison, C. M. & Evans, R. D. Gamma-Ray Absorption Coefficients. *Rev. Mod. Phys.* **24**, 79-107 (1952).

[13] Spotheim-Maurizot, M. *Radiation Chemistry: From Basics to Applications in Material and Life Sciences* (EDP Sciences, Paris, 2008).

[14] de Gennes, P. G. *Scaling Concepts of Polymer Physics* (Cornell University Press, Ithaca, NY, 1979).

[15] Minagawa, K., Matsuzawa, Y., Yoshikawa, K., Matsumoto, M. & Doi, M. Direct Observation of the Biphasic Conformational Change of DNA Induced by Cationic Polymers. *FEBS Lett.* **295**, 67-69 (1991).

[16] Tang, J., Du N., & Doyle, P. S. Compression and self-entanglement of single DNA molecules under uniform electric field. *Proc. Natl. Acad. Sci.* **108**, 16153-16158 (2011).

[17] Terazima, M., Okamoto, K. & Hirot, N. Transient Radiocal Diffusion in Photoinduced Hydrogen Abstraction Reactions of Benzophenone Probed by the Transient Grating Method. *J. Phys. Chem.* **97**, 13387-13393 (1993).

[18] Pimblott, S. M. & LaVerne, J. A. MODELS FOR THE RADIATION CHEMISTRY OF AQUEOUS SOLUTIONS. *Rad. Prot. Dos.* **52**, 183-188 (1994).

[19] LaVerne, J. A. & Pimblott, S. M. Scavenger and Time Dependence of Radicals and Molecular Products in the Electron Radiolysis of Water: Examination od Experiments and models. *J. Phys. Chem.* **95**, 3196-3206 (1991).

[20] Suzuki, M., Crozatier, C., Yoshikawa, K., Mori, T., & Yoshikawa Y. Protamine-induced DNA compaction but not aggregation shows effective radioprotection against double-strand breaks. *Chem. Phys. Lett.* **480**, 113-117 (2009).

[21] Yoshikawa, Y., Mori, T., Magome, N., Hibino, K. & Yoshikawa, K. DNA compaction plays a key role in radioprotection against double-strand breaks as revealed by single-molecule observation. *Chem. Phys. Lett.* **456**, 80-83 (2008).

[22] Zinchenko, A. A., Yoshikawa K. & Baigl D. Compaction of single-chain DNA by Histone-Inspired Nanoparticles. *Phys. Rev. Lett.* **95**, 228101 (2005).

**ACKNOWLEDGMENTS**

We thank M. Ichikawa, Y. Maeda, Y. Yoshikawa, M. Suzuki, S. N. Watanabe S. Hirot, and M. Miyaji for useful discussions. This work was supported by a Sasagawa Scientific Research (Grant No. 24-621) from the Japan Science Society, and partly by Grant-in-Aid for Scientific Research (A) (No. 23240044) from the Japan Society for the Promotion of Science (JSPS).