Thymidine Decomposition Induced by Low-Energy Electrons and Soft X Rays under N$_2$ and O$_2$ Atmospheres

Elahe Alizadeh,a,1,2 Ana G. Sanz,b,2 Guru S. Madugundu,a Gustavo García,a J. Richard Wagnera and Léon Sanchea

a Groupe en Sciences des Radiations, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Canada, J1H 5N4; and b Instituto de Física Fundamental, Consejo Superior de Investigaciones Científicas, Madrid, Spain

INTRODUCTION

High-energy ionizing radiation (α, γ, X rays, protons, heavy ions, etc.) causes a variety of structural lesions to DNA, including single- and double-strand breaks (SSB and DSB), DNA-DNA or DNA-protein crosslinks, base release and chemical modifications of the DNA components (1) which can subsequently lead to cell mutation and death. These molecular lesions are induced either by the direct interaction of radiation with any of the individual DNA moieties (i.e., nucleobases, the sugar or the phosphate group) (2, 3) or alternatively, by the indirect interaction of the reactive species induced from molecules surrounding DNA (4, 5). The latter, commonly referred to as the indirect effect, arises principally from the reaction of water radiolysis products (hydroxyl radicals, solvated electrons and H atoms) with DNA. In both the direct and indirect effect, the energy imparted to the biological media occurs mainly via ionization (6), generating large quantities of secondary species along the radiation track, i.e., ions, radicals and secondary electrons (SEs) (7). SEs are the most abundant secondary species, with about 5 x 10^4 produced per MeV of deposited energy. Typically, SEs have initial kinetic energies lying below 30 eV and a most probable energy of about 9–10 eV (8). These electrons are slowed down by inelastic collisions with the molecules of the media initiating further excitations and ionization processes. Therefore, prior to being thermalized, SEs can induce severe structural and chemical alterations (7). At energies below about 15 eV, electrons can efficiently attach temporarily to particular sites in DNA, leading to the formation of transient negative ions (TNIs) (9). These metastable anions may dissociate into a highly reactive neutral radical and an anion within a very short time period, a reaction known as dissociative electron attachment (DEA). This process plays a crucial role in radiation damage, since it can induce the rupture of chemical bonds. Therefore, mechanisms of low-energy electron (LEE) attachment and subsequent breaks in chemical bonds have been widely investigated experimentally (10) and theoretically (11, 12) by density functional theory treatment of simple models of DNA. Kopyra reported DEA experiments to an entire gas phase nucleotide 2'-deoxyxycytidine 5'-monophosphate (dCMP). They indicated that both direct electron attachment to the backbone and transfer of the excess electron from cytosine to the backbone contributed to SSB formation. Additionally, the majority of SSBs resulted from direct electron attachment to the backbone, since LEEs are more likely captured by the nucleobases rather than other DNA components, due to their higher electronegativity. Thus, TNIs can induce the N-glycosidic (N1–C1') cleavage (13) and therefore, the release of free nucleobases. Alternatively, either by electron transfer from
the base to the phosphate group (14) or through direct electron capture by the phosphate moiety, LEEs can induce cleavage of the C-O bond and thus, SSB and DSB (15). Additionally, the interaction of LEEs with DNA can produce further nucleobase lesions or modifications (16), such as 5,6-dihydrothymine, shown to be a significant radiation-induced product (17).

To gain a better understanding, at the molecular level, of LEE-DNA interactions, measurements of the damage induced to isolated DNA building blocks have shown to be highly valuable. Such investigations have been reported under vacuum conditions, with simple DNA components in the gas-phase and in the condensed-phase in the form of thin molecular films (16, 18). Although all these investigations provided crucial information on the basic mechanisms inducing damage, they could not be directly extrapolated to real cellular conditions, since they were conducted under high vacuum. It is well known (19) that the mechanisms of DNA damage governed by ionizing radiation, and more specifically by LEEs, are strongly affected by the presence of nearby cellular biomolecules, such as O₂.

Recently, a method was developed to allow the investigation of LEE-induced damage to DNA under standard ambient temperature and pressure (SATP) (20, 21). Following this technical advance, numerous studies (18) have reported considerable damage enhancement in DNA samples when irradiated under high concentrations of oxygen. For instance, Alizadeh et al. (21) showed that exposure to an oxygenated atmosphere doubled the damage induced in plasmid DNA by both X rays and LEEs. This radiosensitization is normally attributed to the addition of oxygen to carbon-centered radicals in competition with the chemical repair of radicals by reaction with cellular thiol, a phenomenon known as the oxygen fixation hypothesis (OFH) (22). This hypothesis assumes that O₂ molecules attach to short-lived target radicals, thereby irreversibly fixing the damage via the formation of peroxy radical (DNA-OO·) (23).

Thymidine (C₉H₆N₂O₅; Thd) is a nucleoside composed of a 2-deoxyribose (dR) joined to the nucleobase thymine through the N-glycosidic bond C1–N1. Studies on electron collision involving this nucleoside have been reported by a few groups. Initial gas-phase measurements have been reported by Abdoul-Carime et al. (24) and Ptasińska et al. (25), who showed that LEEs can induce thymidine fragmentation via DEA into low-lying unoccupied π* orbitals. Bald et al. (26) performed similar experiments by means of laser-induced acoustic desorption (LIAD) for thermal evaporation of the samples. More recently, fragments generated from dehydrogenated thymidine have been analyzed with matrix-assisted laser desorption and ionization (MALDI) technique (27). Further vacuum measurements on LEE interaction with Thd in the condensed phase were first provided by Zheng et al. (13) and later by Li et al. (28). Both studies analyzed base release from thymidine solid films by means of high performance liquid chromatography (HPLC) with UV detection and mass spectrometry. In general, there is a lack of experimental data and information about the mechanisms governing the formation of different LEE-mediated Thd products other than base release. Furthermore, except for conformational analysis of DNA (i.e., single- and double-strand breaks) no information presently exists on the products induced by LEE irradiation of any biomolecule at SATP (i.e., under conditions closer to those in cells).

In the current study, we irradiated thin films of thymidine with 1.5 keV X rays under well-controlled environmental conditions with the technique originally applied to plasmid DNA by Alizadeh et al. (21). The Thd samples were deposited onto two different surfaces, i.e., an insulator (borosilicate glass) and a metal (tantalum) substrate. Subsequently, they were irradiated under pure dry N₂ and O₂ at SATP. Our results show the formation of four radiation-induced products, which include free thymine and the following nucleoside modifications: 5-hydroxymethyl-2‘-deoxyuridine (5-HMUrdd), 5-formyl-2‘-deoxyuridine (5-FodUrdd) and 5,6-dihydrothymidine (5,6-DHThd), as inferred by HPLC coupled with tandem mass spectrometry (LC-MS/MS) analysis. These results constitute the first measurement of products induced from LEE irradiation of a biomolecule (e.g., thymidine) at SATP, as well as the comparison of the amount of these products with those generated by soft X rays under the same exact conditions. Since the chamber containing the samples is flushed and enclosed with either nitrogen or oxygen, the effect of O₂ could be examined using the same experimental setup. Interestingly, O₂ increased total LEE damage by factors between 2.4 and 3.5, which is similar to the effect of O₂ observed for cells exposed to ionizing radiation. Although many parameters impact the effect of oxygen on irradiated cells (29), under selected and simplified conditions, we assume that oxygen may have a similar effect in producing damage in plasmid DNA as it does in cellular DNA.

**EXPERIMENTAL METHODS**

**Sample Preparation**

Thymidine was purchased from Sigma-Aldrich (St. Louis, MO) at a stated purity of 99.5% and diluted with distilled and deionized water (ddH₂O) until a final concentration of 50 ng/μL⁻¹ was reached. The solution concentration was more accurately checked by measuring the optical density at 260 nm with a Synergy HT-I spectrophotometer, assuming a molar optical density of 8.41 ODU/μmol at pH 7.0 (30). The purity of Thd solutions was checked with HPLC analysis and showed negligible degradation.

Thd films should be prepared as thin and uniform as possible since photoemitted LEEs have a fairly short range when passing through biological matter. They were prepared following the scheme developed previously for thin DNA films (21, 31). To obtain a fairly uniform
thickness of approximately 10 nm, a 12 µL drop of solution containing 600 ng of pure Thd was deposited on cleaned glass and tantalum substrates. Afterward, samples were frozen at ~70°C and lyophilized. The film thickness was estimated from the mass of the Thd deposited, the surface area of the samples having a ring shape of 7.0 ± 0.2 mm average diameter and the known density of Thd (i.e., 1.45 g/cm³) (32). After this step, HPLC analysis showed that nonirradiated lyophilized Thd samples deposited on both tantalum and glass substrates underwent minimal degradation.

Experimental Setup and Irradiation Conditions

The experimental setup has been previously described in similar studies with DNA films (21, 33) therefore, only a brief outline is given here. The soft X-ray source consists of a stainless steel chamber, which is connected to a Baratron gauge and an adjustable leak valve linked to a nitrogen gas tank. The chamber is evacuated down to ~5 mTorr by means of a mechanical pump. A nitrogen plasma discharge with a 5.5 mA current is established between the cold-cathode and an aluminum foil target in the chamber generating characteristic Kα X rays of 1.5 keV energy. A portion of these X rays, after traversing a flight tube continuously flushed with helium gas and then a thin foil of Mylar, enters into a small chamber filled with dry N₂ or O₂ at atmospheric pressure. The gases have a stated purity of 99% and the small chamber is devoid of humidity, as monitored by a hygrometer. Under such dry conditions, only 2.5 water molecules per nucleotide are assumed to be present in the samples. This water cannot be removed by lyophilization. Within this chamber 18 freeze-dried Thd films can be placed successively in front of the source to be exposed to X rays of varying fluence.

To differentiate damage induced to Thd molecules by either X rays or LEEs, we employed two different substrates, namely, glass and tantalum. During each irradiation cycle, three samples served as controls, they were not exposed to X rays. The remaining samples were exposed in groups of three to X rays for exposures of 2, 4, 7 and 9 h. To measure the number of photon incidence on individual samples, small pieces of GAFCHROMIC HD-810 radiochromatic dosimetry films (Advanced Materials Group of International Specialty Products Technologies Inc., Wayne, NJ) (34) were placed on each plate close to the samples. The interaction of the incident X rays with the tantalum atoms generates energetic SEs within the metal mainly via the photoelectric effect. The energy distribution of SEs generated at the tantalum surface peaks at 1.4 eV with an average energy of 5.85 eV (21). The area under the curve reveals that 95% of the photoelectrons emitted from the metal surface have energies lower than 30 eV. The electron flux and the yield of electron emission per incident photon (nₑ) were taken as (0.54 ± 0.2) × 10⁹ electrons/s/cm² and 0.047 ± 0.005 e/photon, respectively, from a previous study (21).

Sample Recovery and Product Quantification by LC-MS/MS

After X-ray exposure, both irradiated and nonirradiated samples were immediately removed from the chamber and recovered with 30 µL of phosphate buffer (0.4 M, pH 7.0) and 15 µL of ddH₂O in three independent steps (15 µL/each). They were then analyzed by LC-MS/MS. Samples were first subjected to liquid chromatography separation by means of a conventional HPLC apparatus (Shimadzu LC-10 ADvp pumps) equipped with an autosampler, a degasser, column oven (CTO-10ASvp) and UV/Vis detector (SPD-20A) working at 220 and 260 nm. The separation was performed in reversed phase using an ODS-A column (5 µm particle size, 150 length × 2.0 mm inner diameter; YMC). The products were detected at a flow rate of 0.25 mL/min using a linear gradient starting with ammonium formate buffer (5 mM, pH 5.0) containing acetonitrile (ratio of buffer to acetonitrile = 95:5) and going to a higher percentage of acetonitrile (ratio = 80:20) in 10 min, followed by a wash cycle (5 min in 70% acetonitrile and an additional 3 min to re-equilibrate the column). The liquid chromatograph is coupled to an API 3000 tandem mass spectrometer system (MS/MS) through a turbo-ionspray source (MDS SCIEX, Applied Biosystems). The products were detected in the positive ionization mode with a triple-quadrupole system using multiple reaction monitoring (MRM). To minimize the error introduced by the LC-MS/MS analysis, it is customary to add internal standards to the samples. These compounds have the same chemical properties and thus, they were added to the analysis to correct for losses of products during sample preparation and changes in chromatography and electrospray ionization for LC-MS/MS analyses. In the current study, isotopic labeled internal standards for thymidine, thymine, 5-FordUrd and 5-HMdUrd within +2–+4 amu were added to samples after irradiation. Experimental details for the synthesis of labeled standards of 5-FordUrd and 5-HMdUrd, as well as their
calculation curves and analysis in DNA by LC-MS/MS is reported elsewhere (35).

The calibration of thymine was done by the addition of isotopic thymine while simultaneously monitoring the natural and labeled compounds by MRM. The calibration of 5,6-DHThd was carried out by external standard analysis, by means of injecting a known amount of compound in separate chromatographic runs before and after the injection of the sample. For the latter two compounds (thymine and 5,6-DHThd), calibration curves were not recorded over a wide range of concentrations, but the concentration of the standard was prepared to be comparable to the concentration of the product at the highest dose.

The amount of product was determined by comparison of the ion signals for the natural and isotopic compounds during the same chromatographic run. Key parameters for the LC-MS/MS measurement, namely, retention times and fragmentation transitions, are specified in Table 1. The concentration of each Thd radiation-induced product was calculated from the area of the absorbance peak normalized by means of injecting a known amount of compound in the limit of detection for various pyrimidine derivatives.

TABLE 1

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Transition (Da)</th>
<th>Internal standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.6 DHThd</td>
<td>7.5</td>
<td>245.1/117.1</td>
</tr>
<tr>
<td>5-FordUrd</td>
<td>8.0</td>
<td>257.0/141.0</td>
</tr>
<tr>
<td>5-HMdUrd</td>
<td>7.0</td>
<td>259.0/143.0</td>
</tr>
<tr>
<td>Thymine</td>
<td>6.5</td>
<td>127.0/110.1</td>
</tr>
<tr>
<td>Thymidine</td>
<td>8.5</td>
<td>243.3/117.1</td>
</tr>
</tbody>
</table>

To quantify the damage induced by either X rays or LEEs, the damage yields are expressed in terms of the number of damaged molecules per incident photon/cm² for samples deposited on glass [Y_Gl] and tantalum [Y_Gl]. Thus, the number of damaged Thd induced in each film for a given photon fluence (Φ = 10¹² photons/cm²) is given by D_Gl = [Y_Gl] · Φ · N_Thd and D_Gl = [Y_Gl] · Φ · N_Thd where N_Thd is the number of thymidine molecules in each sample, calculated via

\[
N_{Thd} = \frac{m(g) \cdot N_A (mol^{-1})}{M_w (g/mol)} = 1.49 \times 10^{14}
\]

where N_A is the Avogadro’s number and m = 600 ng is the mass of thymidine in each film. Hence the number of absorbed photons in the thymidine film is calculated by

\[
X_{Absorbed} = \Phi \cdot S \cdot \left(1 - e^{-\frac{\mu}{\rho}}\right)
\]

and the number of photons passing through the film to produce photoelectrons at the tantalum substrate by

\[
X_{Transmitted} = \Phi \cdot S \cdot \left(e^{\frac{\mu}{\rho}}\right)
\]

Thus, from our results, the G value for Al Kα X rays is given by

\[
G_X = \frac{D_{Gl}}{1486(eV) \cdot X_{Absorbed}} \cdot 100(eV)
\]

Considering \(\eta_l = 0.047 \pm 0.005\) e/photon as the number of secondary electrons induced by each incident photon (27), by subtracting the damage from tantalum and glass, the G value for LEEs, \(G_{LEE}\), can be calculated by

\[
G_{LEE} = \frac{(D_{Ta} - D_{Gl})}{5.85(eV) \cdot \eta_l \cdot X_{Transmitted}} \cdot 100(eV)
\]

\(G_X\) and \(G_{LEE}\) are calculated within 20% and 25% errors, respectively, which arise mostly from the uncertainty on the area of the DNA film and its thickness and photon fluence, as well as the concentration of sample and \(\eta_l\). It should be remembered that over the spatial variation of the thickness of 10 nm films, spots may exist where the local thickness is smaller than the thermalization distance of LEEs. For local thicknesses smaller than ~10 nm, complete absorption of the energy of LEEs does not occur. Thus, the calculated G values in our 10 nm films are underestimated, since we assume that all the energy of the photoemitted electrons is absorbed in the films. Since this problem was addressed previously, the calculated G values in the current work are corrected according to the method described by Alizadeh and Sanche (37, 38).
RESULTS

The current experimental results allow for the evaluation of damage to Thd molecules from either soft X rays or LEEs and help quantify the formation of four molecular lesions: base release (thymine loss from thymidine) and the following modifications: 5-HMdUrd, 5-FordUrd and 5,6-DHT. Figures 1–3 show the formation of the different nucleoside radiation-induced products relative to undamaged Thd as a function of the X-ray exposure (exposure-response curves) for samples deposited onto both tantalum and glass substrates. Variations in the appearance of these products under N₂ and O₂ atmospheres are shown in Figs. 1–3 (left and right panels, respectively). Each point in the graphs corresponds to the mean value of the yields from three samples exposed to identical conditions. The error bars denote the standard deviation from these means, i.e.,

FIG. 1. Exposure-response curves for the appearance of thymine (base release) formed in thin films of thymidine deposited on either tantalum and glass substrates and irradiated by 1.5 keV X rays under N₂ and O₂ at SATP.

FIG. 2. Exposure-response curves for the appearance of 5-formyl-2'-deoxyuridine (5-FordUrd) and 5-hydroxymethyl-2'-deoxyuridine (5-HMdUrd) in thin films of thymidine deposited on either tantalum and glass substrates and irradiated by 1.5 keV X rays under N₂ and O₂ at SATP.
the statistical error. As expected, we observed that the amount of these products increase with the photon fluence, over the entire range. A detailed analysis of the figures reveals higher yields of damage when the samples are deposited on the tantalum surface, independent of the surrounding atmosphere, in accordance with previous DNA studies (21, 33). This enhancement of nucleoside damage is due to LEEs emitted from the metal surface. Damage yields observed in Thd samples deposited on the glass substrate were induced by the absorbed X rays, while damage yields in the Thd samples deposited on tantalum were induced by both X rays and LEEs (18, 21). The difference in the yields between both substrates can therefore be attributed to the interaction of LEEs with Thd molecules (21). The dose-response curves also show that free thymine, 5-FordUrd and 5-HMdUrd yields are larger under an oxygenated atmosphere, independent of the substrate they are deposited on. In contrast, no 5,6-DHThd was detected in irradiated samples surrounded by O2.

To remove trace amounts of Thd degradation induced by environmental factors other than radiation, we included a set of three control samples both for glass and tantalum substrates in each experimental run, which are represented by the first points in Figs. 1–3. Whereas thymine was not released or lay below the detection limit of the LC-MS/MS system without X-ray exposure, there were traces of 5-FordUrd, 5-HMdUrd and 5,6-DHThd in initial stock solutions of thymidine. Somewhat greater damage was detected for control samples deposited on the tantalum substrates, which can be attributed to the reactivity of the metal surface (21). However, the levels of damage for thymidine were much less in the nonirradiated vs. irradiated samples (~86% and ~88% less 5-FordUrd and 5-HMdUrd were detected in nonirradiated tantalum samples within an O2 atmosphere and up to ~90% less under atmospheric N2).

The data shown in Figs. 1–3 were fitted by a linear least square procedure. The slopes of the lines fitted to the exposure-response curves give the percentage yields of the radiation-induced products formation per photon fluence, for samples deposited on glass (YGl) and tantalum (YTa) and surrounded either by N2 or O2 molecules. Such data, which are summarized in Table 2, represent the rate of formation of thymine, 5-FordUrd, 5-HMdUrd and 5,6-DHThd. Table 2 also provides the G values in nmol/J and D/100 eV for 1.5 keV soft X rays and LEEs, derived from the yields of the specified damages. For a clearer comparison of the effectiveness of X-ray photons and photoemitted LEEs to induce damage, we define an enhancement factor as the ratio of $G_{\text{LEE}}$ to $G_X$.

From the data shown in Table 2, one can find that under N2 atmosphere G values for total damage induced by X rays and LEEs are 95 ± 15 nmol/J and 358 ± 57 nmol/J, respectively, which are comparable to those obtained for loss of supercoiled plasmid DNA in our previous studies, i.e., 98 ± 20 nmol/J and 260 ± 50 nmol/J for X rays and LEEs, respectively (37, 38). More interestingly, Table 2 shows that $G_X$ for base release, i.e., 73.8 ± 15 nmol/J, is in good agreement with the $G$ value for X ray (10 kGy), recently obtained by Park et al. (39) in a study of thin films of TpTpT trinucleotide and determined to be 72 ± 4 nmol/J. It is also comparable with the data obtained by Sharma, Purkayastha and Bernhard (40), who studied extensively the mechanisms of strand breaks due to the direct effect of ionizing radiation. They measured yields of 124 ± 8 nmol/J and 112 ± 28 nmol/J, respectively, for free base release as an indicator of strand breaks in the d(CGCGCG)2 hexamer samples prepared as film or crystal X irradiated at room temperature.

Table 2 further indicates that by changing the atmosphere from N2 to O2, the yields of damage for thymine, 5-FordUrd and 5-HMdUrd become larger. This enhancement is evaluated with the ratio O2/N2, which shows that the formation of these products under an O2 atmosphere is equally favored in tantalum and glass samples, within the experimental uncertainty. Additionally, these data show that LEEs produce an enhancement of base release by factors of
LOW-ENERGY ELECTRON DAMAGE TO THYMIDINE-N$_2$ OR -O$_2$

3.9 and 4.9 relative to X rays under N$_2$ and O$_2$ atmospheres, respectively. Thus, base release enhancement induced by LEEs is higher, but tends to be independent of the surrounding atmosphere, within the experimental uncertainty. In contrast, LEEs enhance the formation of 5-FordUrd and 5-HMdhUrd relative to X rays by a factor of 2.1/1.1 and 2.9/1.2 in each case, under an N$_2$/O$_2$ atmosphere, respectively, i.e., the effectiveness of LEEs to induce these two products is higher under N$_2$ atmosphere than those under O$_2$ atmosphere. More remarkably, our results indicate that formation of 5,6-DHThd was only detected in samples deposited on tantalum and glass substrates and irradiated with 1.5 keV photons under N$_2$ and O$_2$ at SATP.

Since reaction mechanisms of direct damage induced by X rays and LEEs to thymidine are well established (7, 28), these are discussed only briefly here in the context of the current experiment. When 1.5 keV X rays traverse the samples, a portion of them interacts directly with the thymidine molecules, essentially by the photoelectric effect (43). The generated photo/Auger electrons deposit their energy within the Thd molecules leaving them either ionized (∼50%) or electronically excited (8, 44, 45). Secondary LEEs emitted from the metal can also interact directly with Thd and induce additional direct damage. Subsequently, base release and other modifications can occur. LEEs can become trapped in the molecule into low-lying π* orbitals associated with the aromatic ring of the thymine base and form a TNI (46), which can subsequently dissociate into a highly reactive neutral radical and an anion via DEA. This results in a higher number of dissociative states induced by LEEs than via X-ray interaction. LEEs can fragment the glycosidic N1-C1 bond either at subexcitation energies, through transfer of the captured electron to sugar moiety (Reaction 1), or at energies above 5 eV (24, 25). In the latter case, the electron likely remains on the nucleobase (Reaction 2):

\[ \text{Thd} + e^- \rightarrow \text{Thd}^{-*} \rightarrow (\text{Thy} - H)^{-} + (dR \cdot \text{OH})^{-}; \quad (E < 3 \text{ eV}) \]

(1)

\[ \text{Thd} + e^- \rightarrow \text{Thd}^{-*} \rightarrow (\text{Thy} - H)^{-} + (dR \cdot \text{OH})^{-}; \quad (E > 5.5 \text{ eV}) \]

(2)

Alternatively, DEA reactions can induce the loss of a neutral hydrogen radical (H') (47, 48) or ejection of the hydride anions (H') (49, 50), depending also on the incident energy, what may ultimately lead to base modification:

<table>
<thead>
<tr>
<th>Products</th>
<th>Environment</th>
<th>$Y_{el}$</th>
<th>$Y_{ia}$</th>
<th>$G_X$</th>
<th>$G_{1ee}$</th>
<th>$G_X$</th>
<th>$G_{1ee}$</th>
<th>$EF = G_{1ee}/G_X$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymine</td>
<td>N$_2$</td>
<td>2.6 ± 0.2</td>
<td>4.3 ± 0.4</td>
<td>73.8 ± 50</td>
<td>286 ± 57</td>
<td>0.72 ± 0.15</td>
<td>2.8 ± 0.6</td>
<td>3.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>O$_2$</td>
<td>8.4 ± 0.1</td>
<td>15.3 ± 0.2</td>
<td>238 ± 50</td>
<td>1162 ± 232</td>
<td>2.2 ± 0.4</td>
<td>11.5 ± 2.2</td>
<td>4.9 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>O$_2$/N$_2$</td>
<td>3.2 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>3.2 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>0.1 ± 0.02</td>
<td>0.21 ± 0.04</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>5-FordUrd</td>
<td>N$_2$</td>
<td>0.36 ± 0.1</td>
<td>0.49 ± 0.1</td>
<td>10.2 ± 2</td>
<td>21.9 ± 4</td>
<td>0.1 ± 0.06</td>
<td>0.36 ± 0.07</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>O$_2$</td>
<td>1.2 ± 0.1</td>
<td>1.42 ± 0.1</td>
<td>34.1 ± 7</td>
<td>37.1 ± 7</td>
<td>0.3 ± 0.06</td>
<td>0.36 ± 0.07</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>O$_2$/N$_2$</td>
<td>3.3 ± 0.9</td>
<td>2.9 ± 0.6</td>
<td>3.3 ± 0.9</td>
<td>2.9 ± 0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5-HMdhUrd</td>
<td>N$_2$</td>
<td>0.2 ± 0.05</td>
<td>0.3 ± 0.05</td>
<td>5.7 ± 1</td>
<td>16.8 ± 3</td>
<td>0.06 ± 0.01</td>
<td>0.16 ± 0.03</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>O$_2$</td>
<td>0.6 ± 0.1</td>
<td>0.72 ± 0.1</td>
<td>17 ± 3</td>
<td>20.2 ± 4</td>
<td>0.16 ± 0.03</td>
<td>0.2 ± 0.04</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>O$_2$/N$_2$</td>
<td>3.0 ± 0.9</td>
<td>2.4 ± 0.5</td>
<td>3.0 ± 0.9</td>
<td>2.4 ± 0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5,6-DHThd</td>
<td>N$_2$</td>
<td>0.2 ± 0.07</td>
<td>0.4 ± 0.08</td>
<td>5.7 ± 1</td>
<td>33 ± 6</td>
<td>0.06 ± 0.01</td>
<td>0.32 ± 0.06</td>
<td>5.8 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>O$_2$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Note:** The products were obtained from Thd samples deposited on tantalum and glass substrates and irradiated with 1.5 keV photons under N$_2$ and O$_2$ at SATP.

ND = not detected; $EF = G_{1ee}/G_X$.
\[ \text{Thd} + e^- \rightarrow \text{Thd}^{+} \rightarrow (\text{Thd} - H)^+ + H; \quad (E \leq 3 \text{ eV}) \]  

\[ \text{Thd} + e^- \rightarrow \text{Thd}^{+} \rightarrow (\text{Thd} - H) + H^+; \quad (E > 5.5 \text{ eV}) \]

where the release of the hydride anion (H\(^-\)) through DEA occurs mainly at the N3, C6 and the C5-methyl group (50, 51). In the current experiment, all of these DEA reactions are possible, since the energy distribution of the SEs emitted from the tantalum surface ranges essentially from zero to 30 eV and peaks at 1.4 eV, with an average energy ~5.85 eV. Still, it should be noted that SEs in the range between 10–30 eV can ionize thymidine, but this reaction is likely minor in view of the low percentage of electrons within this energy range.

The indirect effects of N\(_2\) and O\(_2\) surrounding DNA have been discussed extensively by Alizadeh et al. (21), so we refer to that work for further details on the species generated by X-ray and LEE interaction with these molecules. Essentially, Al K\(_x\) X rays interact with the N\(_2\) and O\(_2\) molecules mainly through the photoelectric effect and generate highly energetic electrons. A larger amount of photoelectrons are produced within an O\(_2\) atmosphere due to the higher attenuation of oxygen relative to nitrogen (52). These electrons suffer multiple inelastic collisions and in the process, generate numerous reactive species, such as O, O\(^+\), O\(^-\), or O\(_2\)^\(^+\), mainly by either ionization, neutral and dipolar dissociation or DEA (53, 54, 55). When DEA or electron stabilization occurs to produce O\(^+\) or O\(_2\)^\(^+\), respectively, the LEE is scavenged, yielding reactive species. It is important to note that, whereas some of the oxygen products are highly reactive, to form reactive nitrogen species, the presence of oxygen species (O\(_2\) or NO\(_2\)) is required (56). Moreover, Alizadeh et al. (21) reported no detectable enhancement of conformational damage to DNA under nitrogen atmosphere compared to analogous experiments under vacuum. Therefore, we expect no significant Thd damage induced by the indirect effect in nitrogen compared to irradiation in an O\(_2\) atmosphere, where higher amounts of reactive species with larger reactivities are generated. According to the previous DNA experiments, indirect effects of O\(_2\) are equally effective using X and LEE irradiation, within the experimental uncertainty (21). Additionally, one should consider that in general, to observe higher damage yields, oxygen must be present during irradiation or within microseconds after irradiation (57).

**Base Release**

As mentioned previously, the ability of LEEs to induce base release via DEA (Reactions 1 and 2) is well established both in the gas (24, 25) and condensed phase under vacuum (13, 18, 28). In particular, Li et al. (28) irradiated thin Thd films condensed on tantalum substrates with monoenergetic electrons of 11 eV at room temperature. HPLC-UV analysis revealed an average Thd decomposition of 5% of the initial Thd sample, the yield of thymine formation being ~1%. Rupture of the N-glycosidic bond in their study (28) was attributed essentially to reaction 2. In addition, base release may arise from cleavage of C-H bonds at the sugar moiety either by initial capture of LEEs at this site or by transfer of initial TNIs from the base moiety (25). An increase in the percentage of base release from thymidine on glass (2.6%) compared to thymidine on tantalum (4.2%) agrees with a recent study in which solid TpT was exposed to either LEEs under UHV and X rays at SATP (39). Thus, base release is a favored pathway for the reaction of LEEs with DNA components.

Various studies have reported the release of the base thymine from Thd induced directly by photons under vacuum (7, 58, 59). In an early work, Swarts et al. (60) measured the formation of unaltered nucleobases from DNA samples irradiated with \(\gamma\) rays under N\(_2\) and O\(_2\). They found that the yield of free thymine was significantly larger within the latter gas. More recently, Hoffmann and Hütttermann (61) reported thymine release from freeze-dried thymidine-monophosphate samples induced by X rays under nitrogen and air. They observed a linear increase on the yield of free thymine as function of dose, which was ~30% larger on samples irradiated in air composed of 21% of O\(_2\) molecules. Although quantitative comparison is not possible with these studies, they are in qualitative agreement with the current results for samples deposited on glass that show the significant influence of O\(_2\) on the X-irradiated Thd samples (Fig. 1). The effect of O\(_2\) can be explained by its ability to rapidly add to carbon-centered radicals. In particular, the yield of thymine from the exposure of thymidine to ionizing radiation in aqueous solutions is approximately threefold higher in the presence of O\(_2\) compared to that in its absence (i.e., in N\(_2\)-bubbled solution) (62). The exact chemical pathway leading to N-glycosidic bond cleavage by hydroxyl radicals can involve peroxy radicals and various intermediates (63). It is reasonable to suggest a similar pathway to explain base release in the solid state even though hydroxyl radicals are not implicated in the primary step. In the case of direct ionization (i.e., irradiation of thymidine films on glass), base release can be explained by ionization of the sugar moiety followed by deprotonation to give the required carbon-centered radicals at the sugar moiety (64). In the case of LEE induced reactions (i.e., irradiation of thymidine films on tantalum), base release can be explained by the initial formation of TNI at the base moiety followed by transfer of the electron to either the C-N bond or the C-O bond of the sugar moiety. A number of theoretical studies indicate that LEE-induced cleavage of the C-N bond as well as cleavage of the C5'-O5' and C3'-O3' have relatively low activation energies (65–67). If cleavage of the C-O bond occurs, then this will again generate carbon centered radicals at the sugar moiety that can explain the increase in base release in the presence of O\(_2\).
Oxidatively Generated Base Modifications: Formation of 5-FordUrd and 5-HMdUrd

Ionizing radiation can induce the oxidation of Thd to 5-HMdUrd and 5-FordUrd under different conditions, such as in DNA aerated aqueous solutions (68), DNA solid films irradiated with γ rays (69) and also multilayer thymidine films irradiated with soft X rays under vacuum (70). A description of the photooxidation mechanisms leading to the formation of these compounds is available in the literature (71).

Table 2 shows larger $G_{\text{LEE}}$ than $G_{\text{x}}$ for both oxidized nucleosides under both environments, owing to the efficient action of LEEs. The mechanism of LEE-mediated oxidation inducing the formation of 5-formyl-2-deoxyuridine and 5-hydroxymethyl-2-deoxyuridine in thymidine is proposed in Scheme 1, which includes several pathways. The loss of hydride anions (–H–) from the methyl group via DEA of electrons with incident energy of ~10 eV (50) (Reaction 4) is the first step (2 in Scheme 1) of a series of reactions that explains the formation of the oxidized 2-deoxyribonucleoside compounds. The resulting transient compound is a neutral C-centered radical (3), which is highly reactive and may therefore react rapidly with O$_2$ to form a peroxyl radical (ROO$^\cdot$) (4). This radical may be reduced by electron transfer and subsequently undergo protonation (5), leading to the generation of 5-hydroperoxymethyl-2-deoxyuridine (72). This compound is converted into either 5-HMdUrd or 5-FordUrd by dehydration (pathway C) (73). Additionally, the peroxyl radical may eliminate the superoxide radical O$_2^\cdot$ (8), followed by the addition of a H$_2$O molecule giving the alcohol, 5-HMdUrd (6) (pathway B) (74). Alternatively, 5-HMdUrd (6) and 5-FordUrd (7) can be formed through the Russell mechanism (75), i.e., the combination of two 5-(2'-deoxyuridinyl)hydroperoxymethyl radicals (3) may form a tetroxide intermediate (9), which subsequently decomposes into 5-HMdUrd (6) and 5-FordUrd (7), accompanied by the release of O$_2$ (pathway C). It should be noted that the observed increase in the LEE-mediated formation of 5-hydroxymethyl-2'-deoxyuridine and 5-formyl-2'-deoxyuridine could be explained by the reaction of oxygen with 5-(uracilyl)methyl radical once the irradiated sample is dissolved in water.

SCHEME 1. Dissociative electron attachment (DEA) mediated pathways for thymidine (Thd). Pathway A: DEA from the C-N bond leads to immediate base release giving an initial carbon centered radical at C1' of the 2-deoxyribose (dR) moiety and thymine (Thy) anion. When dissolved in H$_2$O, Thy anion undergoes protonation to neutral Thy and the radical gives modified 2-deoxyribose products (73). Pathway B: DEA from the 2-deoxyribose moiety involves cleavage of the C-O bond at either C5' or C3' positions, giving hydroxide ion (OH$^-$) and carbon centered radicals at C5' and C3', respectively. When dissolved in H$_2$O, the latter radicals undergo subsequent reactions that lead to base release (–Thy) together with modified 2-deoxyribose fragment. The yield of base release by pathway B is greater when the irradiation is carried out in O$_2$ compared to N$_2$ because O$_2$ adds to the initial 2-deoxyribose radicals leading to peroxyl radicals and chemistry that facilitates base release. Pathway C: DEA takes place from the exocyclic methyl group of Thd leading to a hydrogen anion (H$^-$) and a 5-(2'-deoxyuridinyl)methyl radical. The latter radical can undergo a number of reactions to explain the formation of 5-HMdUrd and 5-FordUrd. Capture of an O$_2$ molecule gives 5-(2'-deoxyuridinyl)hydroperoxymethyl radical, which can undergo reduction and protonation to give a diamagnetic hydroperoxide. The latter compound can subsequently decompose further either to 5-HMdUrd by reduction or 5-FordUrd by dehydration. Alternatively, a tetroxide intermediate can be formed via the Russell mechanism and subsequently decompose into both 5-HMdUrd and 5-FordUrd. Pathway D: DEA from pathways A to C produce hydrogen atoms (H) or hydrogen anions (H$^-$) that react with Thd to give a 5,6-dihydrothymindin-5-yl radical. The latter radical transforms into either 5,6-DHThd when the irradiation is carried out under a blanket of N$_2$ while this reaction is diverted to the formation of peroxyl radicals and alternative products when O$_2$ is present.
Oxygen-Free Base Modifications: 5,6-dihydrothymidine

Earlier investigations have reported 5,6-dihydrothymidine (5,6-DHThd) to be a major radiation-induced product of thymidine and DNA samples in solid state irradiation with $\gamma$ rays (69, 70) and soft X rays (67) under anoxic conditions, with dose-response profiles showing a linear increase with dose. Additionally, Park et al. (17, 39) explained that the formation of 5,6-DHThd could be mediated by DEA of 10 eV electrons (Reaction 4). The current results corroborate Park and others’ (17) investigation and demonstrate much larger effectiveness of LEEs to induce the formation of 5,6-DHThd compared to 1.5 keV X rays. In this case, formation of 5,6-DHThd can be initiated by the hydride anions (Reaction 4), as proposed by Park et al. (17), and also via hydrogen radicals, which are produced by subexcitation LEEs (Reaction 3). $H^*$ can become attached to the 5,6-double bond of the pyrimidine ring at either the C5 or C6 site. The resulting 5,6-dihydrothymin-5(or 6)-yl radical may be reduced to form 5,6-dihydrothymidine.

More importantly, oxygen is not required for this reaction to take place (70). In fact, no 5,6-DHThd product was detected in Thd films irradiated under an O$_2$ environment. This behavior is expected since the intermediate radical reacts very quickly with O$_2$ molecules, therefore inhibiting the pathway to 5,6-DHThd formation. The final product resulting from the addition of oxygen to 5,6-dihydrothymin-5-yl radicals, such as 5-hydroxy-5,6-dihydrothymidine, was not measured in the current analysis. Additionally, the effect of oxygen may come from the formation of reactive oxygen species by the reaction of LEEs with gaseous oxygen.

CONCLUSIONS

In this article, we reported on the analysis of products induced to thymidine by soft X rays and LEEs under identical conditions at SATP. LEEs were found to be considerably more efficient than X rays in generating radiation-induced products from thymidine, i.e., base release and base modification including 5-HMdUrd, 5-FordUrd and 5,6-DHThd. LEE-mediated damage enhancement tends to be independent of the surrounding atmosphere, within the experimental uncertainty. Among the products analysed, thymine release is the predominant channel, which arises from N-glycosidic-bond cleavage involving $\pi^*$ low-lying TNI. We proposed a LEE-mediated mechanism for the observation of the nucleobase modifications 5-HMdUrd and 5-FordUrd, which involves loss of hydride (−H) from the methyl-group site via DEA. $G$ values derived from these experiments show that formation of free thymine, 5-HMdUrd and 5-FordUrd are favored within an O$_2$ atmosphere compared to a nitrogenous environment, since a larger amount of radicals and ions are formed due to the interaction of radiation with O$_2$, and are in turn, considerably more reactive than those generated under N$_2$. Additionally, O$_2$ can react with C-centered radicals, thereby fixing the damage. In contrast, no 5,6-DHThd was detected when samples were irradiated under an O$_2$ atmosphere, indicating that O$_2$ molecules react with the intermediate radical compound inhibiting the pathway to the formation of 5,6-DHThd. Our study not only provides insight into the well-known radiosensitization mechanism of O$_2$, but also reveals the significant role of LEEs in enhancing the formation of radiation-induced products from thymidine.

ACKNOWLEDGMENTS

This work was funded by the Canadian Institutes of Health Research (CIHR Grant No. MOP 81356). A. G. S. and G. G. acknowledge partial support from the Spanish Ministerio de Economia y Competitividad (Project FIS2009-10245) and the EU COST program (Action MP1002 Nano-IBCT). Financial support was also provided by a discovery grant to JRW from the Natural Science and Engineering Research Council of Canada (NSERC). The authors would like to thank P. Cloutier for technical support.

Received: October 23, 2013; accepted: February 20, 2014; published online: 00 00, 00

REFERENCES


70. Akamatsu K, Fujii K, Yokoya A. Qualitative and quantitative analyses of the decomposition products that arise from the exposure of thymine to monochromatic ultrasoft X-rays and 60Co gamma rays in the solid state. Radiat Res 2004; 161:442–50.


Queries for rare-181-06-12

This manuscript/text has been typeset from the submitted material. Please check this proof carefully to make sure there have been no font conversion errors or inadvertent formatting errors. Allen Press.