

## Indirect Radiation Effects on Thymine in the Glassy State of Its Dilute Aqueous Solution

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Comparable amounts of OH and HO<sub>2</sub> radicals are trapped in hyperquenched glassy water  $\gamma$ -irradiated at 77 K in a dose range up to about 20 kGy. Addition of thymine, in concentration as low as 0.005 M, changes their relative contributions. Thymine radicals (TH) are seen to be formed by addition of atomic hydrogen already at 77 K at the expense of the relative contribution of HO<sub>2</sub> radicals. This is not due to the scavenging of once-formed HO<sub>2</sub> radicals by thymine, but it occurs concurrently with HO<sub>2</sub> radical formation. Therefore, it is rationalized by a concerted process involving the primary intermediate H<sub>2</sub>O<sup>+</sup>. Because of the large water-to-thymine ratio of  $\approx 10^4$  in homogeneous glassy solution, the radiation effect on thymine must be indirect. In contrast, it is known that DNA in freeze-concentrated solution is exposed to either direct or "water-mediated" radiation effects where TH radical formation occurs at much higher temperature.

## Introduction

Radicals generated by high-energy irradiation of liquid water are one of the basic sources of radiation damage to biomolecules.<sup>1</sup> These radicals are short-lived at ambient temperature, therefore they are often studied at cryogenic temperatures by electron spin resonance (ESR) spectroscopy after irradiating either crystalline ice<sup>2</sup> or highly concentrated aqueous electrolyte solution glasses.<sup>3</sup> While these studies provided a wealth of information on, for example, OH and hydroperoxy (HO<sub>2</sub>) radicals, they also bear disadvantages in that further reactions of these radicals with biomolecules may not be those occurring in liquid water, because of formation of other radicals from the solute in the case of the electrolyte solution glass<sup>3</sup> and/or perturbation of the water structure by the solute. Furthermore, in slow-cooled aqueous solutions where ice is formed and phase separation occurs, the OH radicals trapped in the ice compartments are unable to interact with solutes, such as DNA or nucleotides, because these are dissolved only in the "freeze-concentration" regions.<sup>3,4</sup> In freeze-concentrated DNA, radical formation occurs either from DNA by direct interaction with the ionizing radiation or by reaction with radicals generated from water in the hydration shell.<sup>4</sup> In the latter case, the "dry" radicals react with DNA before solvation of the radicals is possible. This is called "water-mediated effect", and it has to be differentiated clearly from indirect radiolysis effects in dilute aqueous solution where the primary radiation products, H<sub>2</sub>O<sup>+</sup> and e<sup>-</sup>, are solvated before further reaction with a solute.

The problems caused by phase separation and freeze-concentration of the solute can be overcome by investigating water and dilute aqueous solutions in their glassy states obtainable by so-called hyperquenching of the liquids.<sup>5–7</sup> In our previous ESR spectroscopic studies of hyperquenched glassy water (HGW) we have shown that, besides OH radicals, which

are the only paramagnetic intermediate of water radiolysis stabilized in irradiated hexagonal ice at 77 K, comparable amounts of HO<sub>2</sub> radicals are trapped in glassy water  $\gamma$ -irradiated at 77 K.<sup>8,9</sup> Surprisingly, the HO<sub>2</sub> radicals are produced in much earlier stages of water radiolysis than expected and they seem to be a primary radiation product.

Here we report an ESR spectroscopic study on  $\gamma$ -irradiation of 0.005 M thymine aqueous solution in the glassy state at 77 K. Thymine was chosen as a solute because the ESR spectra of radicals from thymine are reasonably well characterized,<sup>10</sup> and the low thymine concentration is typically that used for scavengers. We show that a substantial amount of thymine radicals are formed upon  $\gamma$ -irradiation at 77 K at the expense of the relative contribution of HO<sub>2</sub> radicals. This is rationalized by a concerted process involving the primary intermediate H<sub>2</sub>O<sup>+</sup>. Formation of thymine radicals must be an indirect radiation effect because the water-to-thymine ratio is  $\approx 10^4$  in the dilute glassy solution. So far, this primary intermediate was considered to be involved only in the above-mentioned "water-mediated effects".<sup>4</sup> Apparently HO<sub>2</sub> radicals involved in the postulated concerted process are more reactive than OH radicals. This would be significant for further understanding of radiation damage in aqueous solution and of ionizing radiation action in antitumor therapy.<sup>11</sup> So far, OH has been considered to be the most reactive oxygen-containing radical.<sup>12</sup>

## Experimental Section

Thymine (Fluka, Nr. 89270) was used as received. The 0.005 M solution was made with deionized water saturated with gaseous N<sub>2</sub>. As in our previous ESR study of  $\gamma$ -irradiated glassy water,<sup>8,9</sup> the glassy aqueous solution of thymine was prepared by hyperquenching of aerosol droplets on a copper substrate held at 77 K. Solution droplets of  $\approx 5\mu\text{m}$  diameter size made by means of an ultrasonic nebulizer (HICO ULTRASONAT, model 706E, operating at 1.7 MHz, droplet size according to company specifications) were suspended in gaseous nitrogen (99.999%) and passed into a high-vacuum cryostat through a 300  $\mu\text{m}$  diameter aperture. Once inside, the droplets moved at

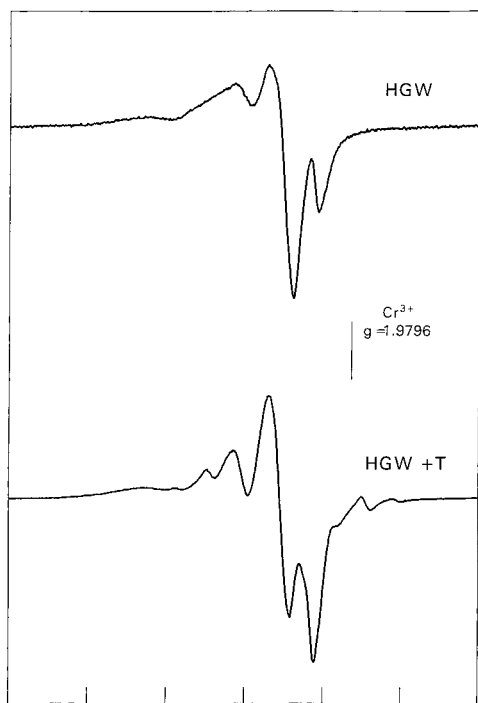
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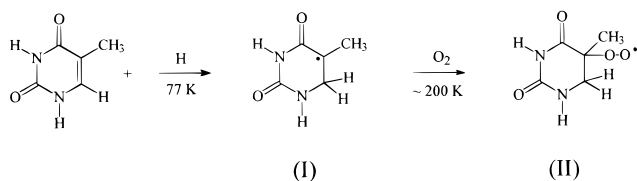
**Figure 1.** ESR spectra recorded at 77 K for hyperquenched glassy water (HGW, top) and hyperquenched glass of 0.005 M aqueous solution of thymine (HGW + T, bottom)  $\gamma$ -irradiated at 77 K.

supersonic speed toward the substrate and deposited on it. All transfers of such samples from one container to the other were made when immersed in liquid  $N_2$ . The  $\gamma$ -irradiations were performed at 77 K in the Co-60 irradiation chamber<sup>13</sup> at a dose rate of about 7.63 kGy/h for 1 h.

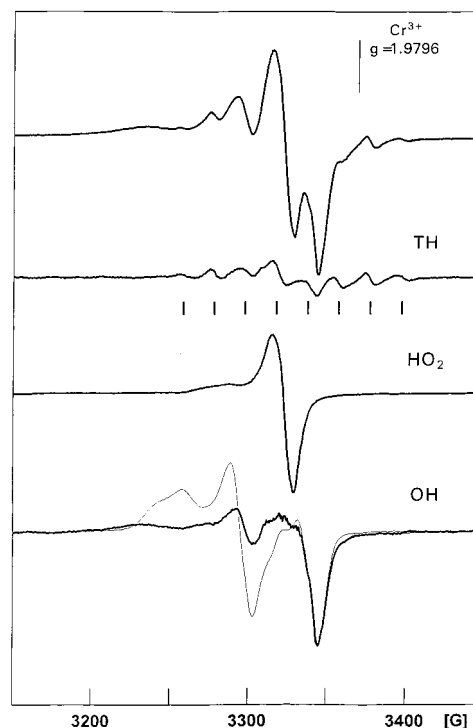
ESR spectra were recorded with an X-band ER 200D-SRC spectrometer, on line with ESP 3220-200SH data acquisition and processing system (BRUKER, Analyt. Messtechnik GmbH). The spectrometer was provided with ESR 900 (Oxford Instruments Ltd.) continuous helium gas flow or a homemade cold nitrogen gas flow cryostat driven by an ITC-4 temperature controller (Oxford Instruments Ltd). For irradiation and measurements at 77 K liquid nitrogen was used as coolant.

## Results

Below, we picture the structure of thymine and of the thymine radicals observed in this study on addition of atomic hydrogen (I) at 77 K and on reaction with  $O_2$  (II) at  $\approx 200$  K.



In Figure 1 we compare the ESR spectrum of  $\gamma$ -irradiated glassy water (HGW, top) with that of a  $\gamma$ -irradiated, 0.005 M glassy aqueous thymine solution (HGW + T, bottom). Both samples were completely vitrified by hyperquenching, the glasses were irradiated at 77 K, and the ESR spectrum was recorded thereafter at the same temperature. The ESR spectrum of HGW was shown to consist of two components of about equal contributions.<sup>8,9</sup> One of them, decaying completely at about 100 K, was identified as the spectrum of OH radicals trapped in glassy matrixes. The other more stable component displayed upon annealing at about 140 K the features charac-

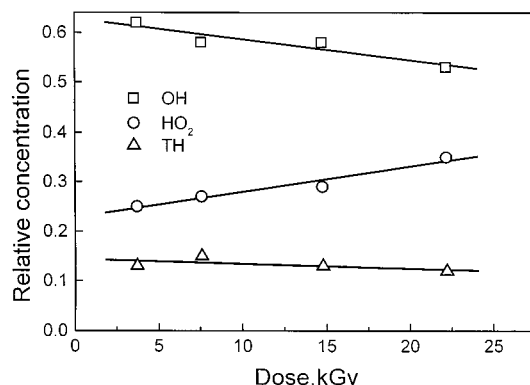


**Figure 2.** Analysis of the ESR spectrum recorded at 77 K for hyperquenched glass of aqueous solution of thymine (0.005 M)  $\gamma$ -irradiated (7.6 kGy) at 77 K. For the relative contributions of TH,  $HO_2$ , and OH radicals see Figure 3. To display the specific features of the OH radical spectrum in aqueous glass, the spectrum of OH radicals recorded at 77 K for  $\gamma$ -irradiated hexagonal ice is depicted (thin line) for comparison.

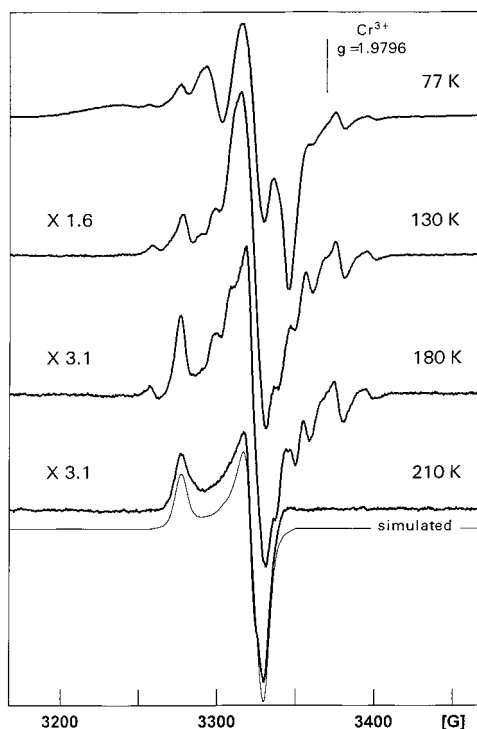
teristic of  $HO_2$  radicals<sup>8</sup> ( $g_x = 2.0040$ ,  $g_y = 2.0075$ ,  $g_z = 2.0355$ ;  $A_x = 5.0$ ,  $A_y = 15.0$ ,  $A_z = 6.0$  G). It decayed at about 150 K.

The ESR spectrum of HGW with 0.005 M thymine also contains the characteristic features of both the OH and  $HO_2$  radicals, although at different ratios in comparison to HGW. In addition, the octet of the 5-thymyl (TH) radicals, an H atom adduct of the thymine molecule at the sixth carbon atom of ring (I),<sup>14</sup> was found in the analysis of the spectrum of the hyperquenched aqueous solution of thymine (see Figure 2). For this analysis the following procedure was followed. First, the ESR spectrum of the TH radical was generated by warming the thymine-containing HGW to 250 K, holding it at 250 K for 10 min to transform quantitatively to hexagonal ice, cooling to 77 K and  $\gamma$ -irradiating at 77 K. At 77 K the ESR spectrum contains the features of OH radicals beside small amounts of TH radicals. On subsequent warming to 140 K, the OH radicals decay, and the ESR spectrum recorded thereafter at 77 K contains only the characteristic octet of the TH radical. Its spectrum was subtracted from that of  $\gamma$ -irradiated thymine-containing HGW. After subtraction of the TH spectrum, the remaining spectrum consisted of OH and  $HO_2$  radicals and it was analyzed exactly as that for neat HGW.<sup>8,9</sup> The three separated components are depicted in Figure 2, and their sum is the original composite spectrum (Figure 2, top). Figure 3 shows the dose dependence of the relative contributions of TH, OH, and  $HO_2$  radicals to the ESR spectra of  $\gamma$ -irradiated HGW containing 0.005 M of thymine.

As expected, the contributions of TH, OH, and  $HO_2$  radicals changed for spectra recorded at 77 K after thermal annealing of the hyperquenched aqueous solution of thymine at subsequently higher temperatures, cf. Figure 4. Only thymine-derived radicals survived the crystallization of HGW into cubic ice. Totally unexpected in deoxygenated system was the appearance



**Figure 3.** Dose dependence of the relative contributions of TH, OH, and HO<sub>2</sub> radicals to the ESR spectra recorded at 77 K of  $\gamma$ -irradiated HGW containing 0.005 M of thymine.



**Figure 4.** ESR spectra recorded at 77 K for the hyperquenched glass of thymine (0.005 M), after  $\gamma$ -irradiation at 77 K and annealing for 10 min at the indicated temperatures. The spectrum recorded after annealing at 210 K has to be recognized as the spectrum of thymine peroxyradicals,<sup>4,15</sup> cf., the spectrum simulated for  $g_x = 2.0022$ ,  $g_y = 2.0076$ , and  $g_z = 2.0349$ .

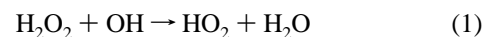
of radicals ( $g_x = 2.0022$ ,  $g_y = 2.0076$ ,  $g_z = 2.0349$ ) to be recognized as the thymine peroxy radicals,<sup>4,15</sup> formed by O<sub>2</sub> attachment to TH radicals at the C5 position (II). After thermal annealing at 210 K the peroxyradicals are the only radicals remaining. These radicals decay upon further annealing at higher temperatures without any detectable transformations.

The shape of the ESR spectrum of glassy water, shown in Figure 1 (top), does not change at 77 K with the dose of  $\gamma$ -irradiation in the range 0.8–18 kGy, which means that the relative contributions of OH and HO<sub>2</sub> radicals remain constant and independent of dose.<sup>8,9</sup> For glassy water containing thymine, cf. Figure 3, the relative contributions of OH, HO<sub>2</sub>, and TH radicals depend moderately on the dose. Their total concentration exceeds that of OH and HO<sub>2</sub> radicals in the neat glassy water. For hexagonal ice, the weak signal of HO<sub>2</sub> radicals starts to build up on top of the spectrum of OH radicals only at doses of hundreds of kGy. These results clearly indicate that HO<sub>2</sub> radicals

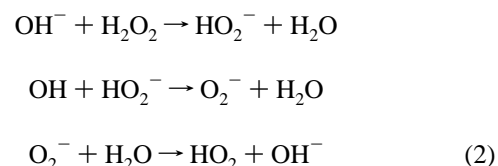
in glassy aqueous systems are formed in much earlier stages of  $\gamma$ -radiolysis than in crystalline or polycrystalline ices.

## Discussion

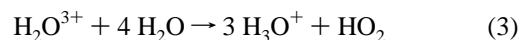
Most probably, OH radicals in hexagonal ice are trapped in the original sites of their formation.<sup>16–18</sup> Part of them are lost, however, presumably via the formation of H<sub>2</sub>O<sub>2</sub>. As the concentration of H<sub>2</sub>O<sub>2</sub> builds up, reaction 1 might also occur,<sup>19</sup> with the formation of HO<sub>2</sub> radicals in low concentration



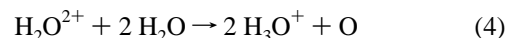
In liquid water, for radiation of high ionization density, the involvement of HO<sub>2</sub><sup>−</sup>, the anion of H<sub>2</sub>O<sub>2</sub> in alkaline solution, has been considered<sup>20</sup> in a series of steps leading to reaction 2



which is considerably faster than reaction 1. To rationalize the high yield of HO<sub>2</sub> radicals in the radiolysis of glassy water by  $\gamma$ -rays it is hard to invoke the process seen<sup>20</sup> under fast-heavy-ion impact. Under these conditions the multiple ionization becomes increasingly important. The triple ionization of water, which necessarily forms a radical species, could directly lead to an HO<sub>2</sub> radical by an acid–base reequilibration, according to the overall reaction

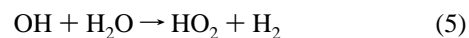


For double ionization of water one would have<sup>20</sup>



followed by the reaction of O atoms with OH radicals to form HO<sub>2</sub>. A weak, but measurable, O<sub>2</sub> yield had been measured for radiolysis of liquid water with <sup>60</sup>Co  $\gamma$ -rays, and this O<sub>2</sub> yield had been assumed to arise from HO<sub>2</sub>.<sup>20</sup>

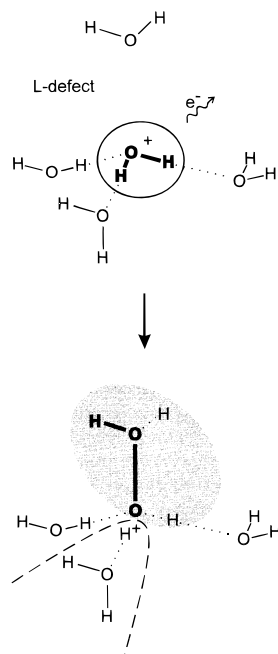
We have sought<sup>8,9</sup> alternative mechanisms such as that shown in reaction 5<sup>21</sup>



However, as it is written, reaction 5 as endothermic, also seemed unlikely. Alternatively, the dissociative recombination of an electron and of H<sub>2</sub>O<sup>+</sup> leading to formation of OH radicals followed by reaction 5 might be considered. This is the pathway recently discussed in low-energy electron-stimulated dissociation of amorphous D<sub>2</sub>O ice to account for atomic deuterium desorption.<sup>22</sup>

We have envisaged a reaction that occurs at shallow proton traps, or so-called Bjerrum-like L-defects.<sup>8,9,23</sup> These defects could be not fully hydrogen-bonded (e.g., three-coordinated) oxygen atoms which we expect to be abundant in glassy water.<sup>8,9,23</sup> The starting point is ionization of a water molecule forming H<sub>2</sub>O<sup>+</sup>. This is the most probable event upon  $\gamma$ -radiolysis.<sup>24</sup> The oversimplification is that in condensed phase the ionization event cannot involve one molecule alone. The H<sub>2</sub>O<sup>+</sup> ion interacts with its neighbors, and the charge must be transferred from one molecule to another.<sup>24,25</sup> Following ionization the required oxygen–oxygen bond can be formed incipiently as a weak 3-electron bond.<sup>26</sup> Because it may be necessary

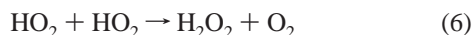
## SCHEME 1



to invoke the participation of excited species for this reaction, we have postulated a concerted process of the type depicted in Scheme 1 (shaded area), with O—O bond formation, loss of H<sub>2</sub>, and formation of H<sub>3</sub>O<sup>+</sup> occurring at about the same time.

For the discussed process, cf., the shaded area in Scheme 1, loss of H<sub>2</sub> seems to be essential. If one of the hydrogen atoms involved in recombination is scavenged, this results in formation of H<sub>2</sub>O<sub>2</sub>. In our scheme the hydrogen atom is scavenged by a thymine molecule leading to the TH (I) radical. Since the water-to-thymine ratio is  $\approx 10^4$ , this must be an indirect radiation effect. Thus HO<sub>2</sub> radicals and TH radicals are formed concurrently. Quantitatively, for 0.005 M thymine glass the relative contribution of OH radicals to HO<sub>2</sub> radicals changes in the range 2.5–1.5, cf., Figure 3, while it was approximately 1:1 in hyper-quenched glassy water.<sup>8,9</sup>

We consider reaction of once-formed HO<sub>2</sub> radicals with thymine unlikely for the following reasons. (i) Although the OH radical generally is more reactive than the HO<sub>2</sub> radical, we observe depletion of HO<sub>2</sub> radical concentration in the presence of thymine, and not that of OH radicals. (ii) Formation of the TH radical from once-formed HO<sub>2</sub> requires splitting of the H—O<sub>2</sub> bond. In this process O<sub>2</sub> is formed also. It is difficult to understand why this O<sub>2</sub> does not react immediately at 77 K with TH, forming the peroxy radical. Its formation occurs only on heating to >160 K (see Figure 4), presumably in the secondary reaction of thymine radicals with O<sub>2</sub> which is formed during HO<sub>2</sub> radical disproportionation (eq 6).



Thus we conclude that at 77 K formation of the TH radical from HO<sub>2</sub> must have occurred in a concerted process. TH formation in freeze-concentrated solution or in hydrated samples occurs as a secondary process at much higher temperatures.<sup>15</sup>

One of the surprising aspects of this study is that the thymine anion radical was not observed. This radical could be formed by attachment of the electron generated in the primary ionization event. It is well-known that thymine is the eventual sink of the long-range electron migration in DNA.<sup>4</sup> The absence of the thymine anion radical could be due to its immediate protonation

and formation of TH radicals by surrounding water molecules. This would be an alternative mechanism. However, in this case the depletion of HO<sub>2</sub> radical relative concentration cannot be understood.

So far, indirect radiation effects in dilute aqueous solution involve the radiation chemistry of H, OH, and e<sup>-</sup><sub>aq</sub> radicals.<sup>4</sup> We suggest that, in addition, the radiation chemistry of the HO<sub>2</sub> radical, formed via our postulated concerted process, should be considered.

It seems interesting to note that preferential loss of molecular hydrogen is seen to enhance the stability of species such as OH, HO<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>, or O<sub>2</sub> in water ice radiolyzed on outer solar system bodies.<sup>27</sup> Also, in the above-mentioned studies<sup>22</sup> on low-energy electron-stimulated dissociation of amorphous D<sub>2</sub>O ice, an important observation was that formation of atomic oxygen must occur by a pathway which involves simultaneous formation of molecular hydrogen.

## Conclusions

The mechanism envisaged for reaction 2, cf., Scheme 1, to account for HO<sub>2</sub> radical formation in early stages of water radiolysis in the glassy state seems to be valid for rationalization of indirect radiation effects on thymine in the glassy state of its dilute aqueous solution. The thymine radicals, TH, an H atom adduct to a thymine molecule, are formed in a concerted process at the expense of HO<sub>2</sub> radicals. Reaction with once-formed HO<sub>2</sub> radicals is unlikely. Loss of H<sub>2</sub> from the reactive complex seems to be essential for formation of HO<sub>2</sub> radicals. Scavenging of one of the two hydrogen atoms involved in formation of molecular hydrogen leaves H<sub>2</sub>O<sub>2</sub> in the reaction center.

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