Steady State Radiolysis of Aqueous Aerated Solutions of 5,6-Dihydrothymine. Identification of the Major Degradation Products

J. Cadet*

Département de Recherche Fondamentale, Laboratoire de Radiobiologie, Centre d'Études Nucléaires, 85 X 38041 Grenoble Cedex, France

Z. Naturforsch. 35b, 1579–1583 (1980); received May 9, 1980

5,6-Dihydrothymine, Hydroperoxides, Dihydropyrimidyl Radicals

The major radiation-induced degradation products of 5,6-dihydrothymine (1) in aqueous solutions saturated either with O₂ or N₂O/O₂ (75/25 v/v) were identified as thymine (10), the trans- and cis-isomers of 6-hydroperoxy-5,6-dihydrothymine (5, 7), 5-hydroperoxy-5,6-dihydrothymine (8), and their corresponding hydroxy derivatives (4, 6, 8). The formation of these compounds is explained in terms of initial H-abstraction from the C(5) and C(6) carbons which gives rise respectively to the transient 5-yl and 6-yl radicals. Thymine hydroxy-hydroperoxides (11–14) and their decomposition products (17–20), which are derived from hydroxyl radical attack at the 5,6-ethylenic bond of thymine (10), were also characterized.

Introduction

Dihydropyrimid-5 and 6-yl radicals have been shown to be produced, in addition to hydroxy-pyrimidine adducts, by gamma irradiation of 2,4-dioxopyrimidines in oxygen free aqueous solution [1, 2]. Hydrogen atoms derived from the radiolysis of water appear to react preferentially at the C(6) carbon [1], whereas protonation of the ketyl radicals [2–4] consecutive to the interaction of solvated electrons with the C(4) carbonyl group has been suggested as the sequence of reactions giving rise to the C(6) radical [2]. Attention has been focused on the pulse radiolysis studies of 5,6-dihydrothymine (1) [2, 5–8] which have been reported to lead to a more selective formation of the 6-yl radical (3) [2, 6]. Further support for the occurrence of preponderant H-abstractions from the C(6) carbons of 1 and 5,6-dihydro uracil by •OH radicals in aqueous solutions has been given by spin-trapping experiments [9]. The formation of the 5-yl radical (2) has been postulated on the basis of the shoulder which was observed on the end absorption (λ ~ 300 nm) in the transient spectrum obtained by pulse radiolysis of these dihydropyrimidines [8].

However, only a few attempts have been made to isolate and characterize the final products arising from radical or ionic transformations of these reactive intermediates. Evidence of the partial conversion of 1 to thymine by gamma irradiation in aqueous solution has been given by a biochemical approach using an E. coli thymine-requiring auxotroph mutant [10]. Organic peroxides have been detected by colorimetry in aerated aqueous solutions of 5,6-dihydrouracil exposed to ionizing radiations [11]. The main stable products, resulting probably from the hydrolytic decomposition of these hydroperoxide precursors, have been identified as barbituric acid and 6-hydroxy-5,6-dihydro uracil [12], suggesting a preferential initial OH- attack at the six position.

The main purpose of the work described in this paper is to assign chemical structures to the main radiation-induced degradation products of 5,6-dihydrothymine (1) in aqueous oxygenated solution. The identification of the two isomers of 6-hydroperoxy-5,6-dihydrothymine (5, 7) and of 5-hydroperoxy-5,6-dihydrothymine (9) gives further support to the hypothesis of hydrogen abstraction from both carbons C-5 and C-6. The relative yields of the various degradation products were not markedly affected by converting most of the solvated electrons to OH radicals, hence partly preventing the formation of superoxide anion radicals, in irradiated solutions of 1 saturated with N₂O/O₂ (75/25 v/v). These results, in agreement with earlier findings [13], suggest that dismutation reactions between peroxo radicals could be involved in the formation of pyrimidine hydroperoxides.

Results

Identification of the products

The gamma irradiation of aqueous solutions of [14C-2] dihydro-5,6-thymine (1 mM) in the presence of water appear to react preferentially at the C(6) carbon [1], whereas protonation of the ketyl radicals [2–4] consecutive to the interaction of solvated electrons with the C(4) carbonyl group has been suggested as the sequence of reactions giving rise to the C(6) radical [2]. Attention has been focused on the pulse radiolysis studies of 5,6-dihydrothymine (1) [2, 5–8] which have been reported to lead to a more selective formation of the 6-yl radical (3) [2, 6]. Further support for the occurrence of preponderant H-abstractions from the C(6) carbons of 1 and 5,6-dihydro uracil by •OH radicals in aqueous solutions has been given by spin-trapping experiments [9]. The formation of the 5-yl radical (2) has been postulated on the basis of the shoulder which was observed on the end absorption (λ ~ 300 nm) in the transient spectrum obtained by pulse radiolysis of these dihydropyrimidines [8].

However, only a few attempts have been made to isolate and characterize the final products arising from radical or ionic transformations of these reactive intermediates. Evidence of the partial conversion of 1 to thymine by gamma irradiation in aqueous solution has been given by a biochemical approach using an E. coli thymine-requiring auxotroph mutant [10]. Organic peroxides have been detected by colorimetry in aerated aqueous solutions of 5,6-dihydrouracil exposed to ionizing radiations [11]. The main stable products, resulting probably from the hydrolytic decomposition of these hydroperoxide precursors, have been identified as barbituric acid and 6-hydroxy-5,6-dihydro uracil [12], suggesting a preferential initial OH- attack at the six position.

The main purpose of the work described in this paper is to assign chemical structures to the main radiation-induced degradation products of 5,6-dihydrothymine (1) in aqueous oxygenated solution. The identification of the two isomers of 6-hydroperoxy-5,6-dihydrothymine (5, 7) and of 5-hydroperoxy-5,6-dihydrothymine (9) gives further support to the hypothesis of hydrogen abstraction from both carbons C-5 and C-6. The relative yields of the various degradation products were not markedly affected by converting most of the solvated electrons to OH radicals, hence partly preventing the formation of superoxide anion radicals, in irradiated solutions of 1 saturated with N₂O/O₂ (75/25 v/v). These results, in agreement with earlier findings [13], suggest that dismutation reactions between peroxo radicals could be involved in the formation of pyrimidine hydroperoxides.

Results

Identification of the products

The gamma irradiation of aqueous solutions of [14C-2] dihydro-5,6-thymine (1 mM) in the presence of water appear to react preferentially at the C(6) carbon [1], whereas protonation of the ketyl radicals [2–4] consecutive to the interaction of solvated electrons with the C(4) carbonyl group has been suggested as the sequence of reactions giving rise to the C(6) radical [2]. Attention has been focused on the pulse radiolysis studies of 5,6-dihydrothymine (1) [2, 5–8] which have been reported to lead to a more selective formation of the 6-yl radical (3) [2, 6]. Further support for the occurrence of preponderant H-abstractions from the C(6) carbons of 1 and 5,6-dihydro uracil by •OH radicals in aqueous solutions has been given by spin-trapping experiments [9]. The formation of the 5-yl radical (2) has been postulated on the basis of the shoulder which was observed on the end absorption (λ ~ 300 nm) in the transient spectrum obtained by pulse radiolysis of these dihydropyrimidines [8].

However, only a few attempts have been made to isolate and characterize the final products arising from radical or ionic transformations of these reactive intermediates. Evidence of the partial conversion of 1 to thymine by gamma irradiation in aqueous solution has been given by a biochemical approach using an E. coli thymine-requiring auxotroph mutant [10]. Organic peroxides have been detected by colorimetry in aerated aqueous solutions of 5,6-dihydrouracil exposed to ionizing radiations [11]. The main stable products, resulting probably from the hydrolytic decomposition of these hydroperoxide precursors, have been identified as barbituric acid and 6-hydroxy-5,6-dihydro uracil [12], suggesting a preferential initial OH- attack at the six position.

The main purpose of the work described in this paper is to assign chemical structures to the main radiation-induced degradation products of 5,6-dihydrothymine (1) in aqueous oxygenated solution. The identification of the two isomers of 6-hydroperoxy-5,6-dihydrothymine (5, 7) and of 5-hydroperoxy-5,6-dihydrothymine (9) gives further support to the hypothesis of hydrogen abstraction from both carbons C-5 and C-6. The relative yields of the various degradation products were not markedly affected by converting most of the solvated electrons to OH radicals, hence partly preventing the formation of superoxide anion radicals, in irradiated solutions of 1 saturated with N₂O/O₂ (75/25 v/v). These results, in agreement with earlier findings [13], suggest that dismutation reactions between peroxo radicals could be involved in the formation of pyrimidine hydroperoxides.

Results

Identification of the products

The gamma irradiation of aqueous solutions of [14C-2] dihydro-5,6-thymine (1 mM) in the presence of
Fig. 1. Two-dimensional thin layer chromatography on cellulose plate of the radiation-induced degradation products of 5,6-dihydrothymine (1) in O₂-N₂O aqueous solution.

1: 5,6-dihydrothymine; 2: thymine; 3, 4: unknown compounds; 5: trans-6-hydroperoxy-5,6-dihydrothymine; 6: trans-6-hydroxy-5,6-dihydrothymine; 7: cis-6-hydroperoxy-5,6-dihydrothymine; 8: 5-hydroxy-5,6-dihydrothymine; 9: cis-6-hydroxy-5,6-dihydrothymine; 10: 5-hydroperoxy-5,6-dihydrothymine; 11: cis-5,6-dihydroxy-5,6-dihydrothymine; 12: trans-5,6-dihydroxy-5,6-dihydrothymine; 13: 5-hydroxy-methyluracil; 14: cis-5-hydroperoxy-6-hydroxy-5,6-dihydrothymine; 15: cis-6-hydroperoxy-5-hydroxy-5,6-dihydrothymine; 16: trans-5-hydroperoxy-5-hydroxy-5,6-dihydrothymine and 6-hydroperoxy-5-hydroxy-5,6-dihydrothymine.

either of oxygen or of N₂O/O₂ (75/25 v/v) gave in both cases similar sets of degradation products. Most of these compounds were separated by two-dimensional thin-layer chromatography on precoated cellulose plates [14], and detected by autoradiography as illustrated in Fig. 1.

Thymine, which was previously identified as the main stable product of the radiation-induced degradation of 1 (Fig. 2) in aqueous oxygenated solution [7, 10] was unambiguously characterized by comparison of its UV, IR and mass spectra with those of an authentic sample. A second UV absorbing compound, which was produced only in low yield in N₂O/O₂ saturated solution was assigned as 5-hydroxymethyluracil.

A second class of four products which exhibited the chemical properties of pyrimidine “hydrates” [15, 16] was identified as the trans and cis isomers of 6-hydroxy-5,6-dihydrothymine (4, 6) and 6-hydroperoxy-5,6-dihydrothymine (5, 7) (Fig. 3) on the following basis. The quantitative dehydration of the “hydrates” (4, 6) in acidic solution (HCl N) is consistent with their quality of β-ketols [17]. Conversion of the hydroperoxides (5, 7) to the corresponding alcohols (4, 6) constitutes the first step of their heat or alkaline catalysed transformation to thymine [18]. The assignment of a trans-configuration for 4 was based on the low magnitude of the ¹H NMR coupling between the vicinal protons H(5) and H(6) (J₅-H₆ = 1.9 Hz) which adopt a preferential diequatorial conformation in DMSO d₆ [19]. The downfield shift of the methyl carbon (−2.5 ppm) in the ¹³C NMR spectrum of the cis-isomer 6 is characteristic of the occurrence of a γ-gauche-interaction between the methyl and hydroxyl groups [20]. Reduction of the hydroperoxides 5, 7 by KI which takes place according to a SN₂ mechanism at the peroxidic bond [21] gave specifically the corresponding hydrates (4, 6) with retention of the C(6) configuration [19]. These products (4–7), 5-hydroperoxy-5,6-dihydrothymine (9) and its stable alcohol derivative (8) are formed in low yield by gamma radiolysis of aerated aqueous solutions of thymine [22].

Fig. 2. Formation of 5-yl and 6-yl radicals.

Fig. 3. Chemical structure of the monosubstituted radiation-induced derivatives of 5,6-dihydrothymine.
In addition to the above major hydroperoxides (5, 7) and (9), the four cis- and trans-6-hydroperoxy-5-hydroxy- and 5-hydroperoxy-6-hydroxy-5,6-dihydrothymines (11-14) were characterized in the irradiated solution of 1 saturated with N2O/O2. Comparison of their chromatographic and chemical properties with those of synthesized thymine hydroxy hydroperoxides [23, 24] were used for their assignment. The main degradation products of these relatively unstable peroxides were identified as cis- and trans-5,6-dihydroxy-5,6-dihydrothymine (15, 16), 5-hydroxy-5-methyl-barbituric acid (17), 5-hydroxy-5-methyl hydantoin (18), N-formyl-5-hydroxy-5-methyl hydantoin (19) and N-acetyl urea (20) on the basis of their IR, 1H NMR and mass spectrometry data [25]. These compounds were produced by radiation-induced degradation of 1 in the presence either of O2 or N2O/O2.

### Table I. G values of the radiation-induced degradation products of 5,6-dihydrothymine (1) in aqueous solution.

<table>
<thead>
<tr>
<th>Products</th>
<th>O2</th>
<th>N2O/O2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,6-Dihydrothymine (1)</td>
<td>1.99</td>
<td>4.10</td>
</tr>
<tr>
<td>trans-6-Hydroxy-5,6-dihydrothymine (4)</td>
<td>0.09</td>
<td>0.20</td>
</tr>
<tr>
<td>cis-6-Hydroperoxy-5,6-dihydrothymine (5)</td>
<td>0.13</td>
<td>0.29</td>
</tr>
<tr>
<td>cis-6-Hydroperoxy-5,6-dihydrothymine (6)</td>
<td>0.10</td>
<td>0.22</td>
</tr>
<tr>
<td>cis-6-Hydroperoxy-5,6-dihydrothymine (7)</td>
<td>0.08</td>
<td>0.61</td>
</tr>
<tr>
<td>6-Hydroperoxy-5,6-dihydrothymine (8)</td>
<td>0.09</td>
<td>0.17</td>
</tr>
<tr>
<td>5-Hydroperoxy-5,6-dihydrothymine (9)</td>
<td>0.24</td>
<td>0.38</td>
</tr>
<tr>
<td>Thymine (10)</td>
<td>0.46</td>
<td>0.50</td>
</tr>
<tr>
<td>cis-6-Hydroperoxy-5-hydroxy-5,6-dihydrothymine (11)</td>
<td>–</td>
<td>0.08</td>
</tr>
<tr>
<td>trans-6-Hydroperoxy-5-hydroxy-5,6-dihydrothymine (12)</td>
<td>–</td>
<td>0.15</td>
</tr>
<tr>
<td>cis-5-Hydroperoxy-6-hydroxy-5,6-dihydrothymine (13)</td>
<td>–</td>
<td>0.02</td>
</tr>
<tr>
<td>trans-5-Hydroperoxy-6-hydroxy-5,6-dihydrothymine (14)</td>
<td>–</td>
<td>0.05</td>
</tr>
<tr>
<td>cis-5,6-Dihydroxy-5,6-dihydrothymine (15)</td>
<td>0.23</td>
<td>0.52</td>
</tr>
<tr>
<td>trans-5,6-Dihydroxy-5,6-dihydrothymine (16)</td>
<td>0.18</td>
<td>0.28</td>
</tr>
<tr>
<td>5-Hydroxy-5-methylbarbituric acid (17)</td>
<td>0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>5-Hydroxy-5-methyl hydantoin (18)</td>
<td>0.01</td>
<td>0.10</td>
</tr>
<tr>
<td>N-Formyl-5-hydroxy-5-methyl hydantoin (19)</td>
<td>0.03</td>
<td>0.24</td>
</tr>
<tr>
<td>N-Acetylmorone (20)</td>
<td>0.01</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* Dose: 115 krad/s with a dose rate = 11.5 krad/s;  
  a N2O/O2 (75/25 v/v) under 1 atmosphere.

### Quantitative results

The yields of formation of the monosubstituted derivatives of 1 as function of the dose were linear in a dose range of 50 to 100 krad/s. The G-values* of the main radiation-induced degradation products of 5,6-dihydrothymine (1) in aqueous solution saturated with O2 or N2O/O2 are listed in Table I.

### Discussion

The radiation-induced degradation of 5,6-dihydrothymine (1) in dilute aqueous solution proceeds essentially through indirect processes. The bulk of the OH- radicals which derive from the gamma radiolysis of water react with 1, the various estimated values of the second order rate constant for this reaction falling in the range 2.2 x 10^9 to 2.5 x 10^9 M^-1 sec^-1 [5, 8]. Hydrogen atoms, which are reported to be six times less reactive than OH- with 1 [8], are mostly scavenged by molecular oxygen. Additional formation of superoxide anion radicals is expected from the predominant reaction of e^-aq with O2 in aqueous oxygenated solutions giving a total G (O2^-) value close to 3.2. In oxygenated solutions saturated with N2O most of the e^-aq are converted to OH- radicals [26] (reaction (1)).

\[
e^{-aq} + N_2O \rightarrow OH- + OH- + N_2 \]  

(1)

thereby preventing partly the formation of O2^- (G ~0.5).

5,6-dihydrothymyl (2) and 5,6-dihydrothymyl-6-yl (3) radicals

The identification of 5-hydroperoxy-5,6-dihydrothymine and the isomers of 6-hydroperoxy-5,6-dihydrothymine (5, 7) among the radiation-induced degradation of 1 in aqueous solution saturated with either O2 or N2O/O2 provides further support for the earlier suggestions that hydrogen abstraction may occur from both the C(5) and C(6) carbons of 1. The resulting 5-yl (2) and 6-yl (3) radicals (Fig. 2) react rapidly with molecular oxygen to give the corresponding peroxo radicals (reaction (2)) as demonstrated by pulse radiolysis studies [7, 13].

\[
TH^+ + O_2 \rightarrow 'OOTH  
k = 1.3 \times 10^9 M^{-1}sec^{-1} \]  

(2)

From these 'OOTH radicals two main competitive reactions may explain, in agreement with previous

* G value = number of molecules produced or destroyed per 100 eV absorbed.
pulse radiolysis experiments [7, 13], the formation of the final degradation products.

Hydroperoxides

The involvement of superoxide radicals (reaction (3)) in dismutation reactions with peroxy radicals has been suggested [27] to rationalize the formation of radiation-induced pyrimidine hydroperoxides in aqueous oxygenated solutions.

$$\cdot\text{OOTH} + \cdot\text{O}^- \xrightarrow{H^+} \text{HTOOH} + \text{O}_2 \quad (3)$$

The yield of formation of the hydroperoxides appears to be independent of the steady-state concentration of the superoxide anion radicals (Table I). Similar observations have been made when aqueous oxygenated solutions of thymine were irradiated in the presence of N₂O [13, 28]. Furthermore the sum of the G values for the hydroperoxides (5, 7, 9) and the hydroxy hydroperoxides (11–14) resulting from the degradation of thymine (10) is higher (G = 1.87) than the expected amount of superoxide anion radicals (G ≈ 0.5) in aerated aqueous solutions saturated with N₂O. Finally, additional support for the occurrence of a dismutation reaction [13] between \( \cdot\text{OOTH} \) radicals (reaction (4)) is given by the observation of an approximately 1/1 ratio between the hydroperoxides (5, 7, 9) and the corresponding hydroxy derivatives (4, 6) and (8).

$$2 \cdot\text{OOTH} + \text{H}_2\text{O} \rightarrow \text{HOOTH} + \text{HOTH} \quad (4)$$

Thymine

The formation of thymine (10) and its oxidation products represents about 50% of the total product distribution in both O₂ and N₂O/O₂ (saturated) aqueous solutions of 1 exposed to γ-rays (Table I). Tetroxides, which could result from the self reaction of peroxy radicals (reaction (5)), have been postulated as the precursors of thymine (7).

$$2 \cdot\text{OOTH} \rightarrow \text{HTOOOH} \quad (5)$$

$$k = 6 \times 10^6 \text{M}^{-1}\text{sec}^{-1}$$

It may be suggested, from the above considerations of hydroperoxide formation and product distribution, that the decomposition of this unstable intermediate gives quantitatively 10. The Russell mechanism [29, 30], which invokes the dissociation of tetroxides through a cyclic transition state, is unlikely under our conditions since one of the expected products of this reaction, 5-methylbarbituric acid, is not formed. In conclusion it should be emphasized that the G value for the destruction of 1 in O₂ or N₂O/O₂ saturated solution parallels the theoretical value of G (OH) assuming that the formation of thymine degradation products (17–20) requires two hydroxyl radical attacks.

Experimental

Irradiation procedures

Unbuffered solutions (pH = 6.0) of 10⁻³⁰M [¹⁴C-2] 5,6-dihydrothymine in tripily distilled water were saturated either with O₂ or N₂O/O₂ (75/25 v/v) prior to being exposed to γ-rays from a ⁶⁰Co source. The streams of gases were maintained during the irradiation.

Chemicals

Thymine was purchased from Sigma (St. Louis, Mo., U.S.A.) and the [¹⁴C-2] labelled thymine was a product of the Commissariat a l’Energie Atomique, France. [¹⁴C-2] 5,6-dihydrothymine was prepared by catalytic hydrogenation [31] of a mixture of cold and [¹⁴C-2] labelled thymine. A solution of 40 mg and 40 μCi of thymine in 20 ml of water was hydrogenated in a Parr apparatus at an initial pressure of 40 atmospheres for 4 h in the presence of 5% rhodium on alumina. The catalyst was removed by filtration on a Celite pad and the filtrate evaporated to dryness. Semi-preparative thin-layer chromatography was conducted on three precoated cellulose plates (Merck, Darmstadt, G.F.R.) with 75/16/9 ethyl acetate-2-propanol-water (solvent II) as the developer. The main radioactive zone which was detected by autoradiography (Rf = 0.42) was extracted with methanol (3 × 10 ml). Evaporation of the solution to dryness gave a syrup which was recrystallized from methanol to give a 50% yield of 5,6-dihydrothymine (1) as colourless needles; m.p. 262 °C, lit. [32] 262.5–263 °C.

cis-6-Hydroxy-5,6-dihydrothymine (6) was prepared by mild reduction of trans-5-bromo-5-hydroxy-5,6-dihydrothymine [33]. The trans-isomer (4), trans- and cis-6-hydroperoxy-5,6-dihydrothymine (5, 7) were obtained by peroxidation of 6 [19]. cis-6-Hydroperoxy-5-hydroxy-5,6-dihydrothymine was synthesised by treatment of cis-thymine diol (15) with acidic hydrogen peroxide [24, 34]. 5-Hydroxy-5,6-dihydrothymine (8) [35], cis- and trans-5-hydroperoxy-6-hydroxy-5,6-dihydrothymine (13, 14) [24], cis- and trans-5,6-dihydroxy-5,6-dihydrothymine (15, 16), 5-hydroxy-5-methyl barbituric acid (17) [36], 5-hydroxy-5-methyl hydantoin (18) [25] and N-formyl-5-hydroxy-5-methyl hydantoin [25] were prepared according to literature procedures.
**Spectroscopic measurements**

IR spectra were obtained on a Perkin Elmer, Model 177 Instrument. NMR spectra were registered on a Varian Associates, Model T 60 Instrument operating at 60 MHz. High resolution mass spectra were obtained on a MS 50 mass spectrometer using an ionization voltage of 70 eV. Melting points were taken on a Reicher hot stage apparatus without correction.

**Thin layer chromatography**

Thin-layer chromatography (TLC) was performed on Merck precoated cellulose plates (~0.1 mm thickness) using the lower phase of a mixture of chloroform–methanol–water (4/2/1 by volume) to which 5 ml of methanol was added for 100 ml of the organic layer (solvent I) and ethyl acetate–2-propanol–water (75/16/9 by volume) (solvent II), as the developing solvents.

**Quantitative measurements**

Labeled compounds were localised on the chromatogram after two dimensional thin-layer chromatography (solvents I and II) by exposure to X-ray film (Kodirex) overnight. The radioactive zones of cellulose were scraped off and the labeled material eluted in the counting vial with water. Radioactivity was measured using a Packard Model 2425 Tri Carb® apparatus.