Radiolysis of Cytosine, 5-Methyl Cytosine and 2'-Deoxyctydine in Deoxygenated Aqueous Solution
A Pulse Spectroscopic and Pulse Conductometric Study of the OH Adduct*

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Cytosine, G-Values, pK-Values, Absorption Spectra, Pulse Radiolysis

Using the pulse radiolysis technique the OH adducts of cytosine, 5-methyl cytosine and 2'-deoxyctydine were investigated in alkaline N₂O saturated aqueous solutions. Absorption spectra were recorded and their change with time was correlated with the change in conductivity. An OH⁻ induced reaction (1.4 • 10⁸ 1 • mol⁻¹ • s⁻¹) was observed with OH adducts of cytosine and 5-methyl cytosine leading to radicals (pK = 10.3), the G-values of which were 3.2 and 1.6 respectively (conductivity measurements). These intermediates were assigned to the C-5 OH radical adducts.

After completion of the free radical reactions (2k = 6.5 • 10⁸ 1 • mol⁻¹ • s⁻¹) the resulting product mixture showed pK-values of 8.3 (cytosine, G = 0.6), 10.5 (5-methyl cytosine, G = 0.8), 10.7 (cytosine, G = 1.5; 2'-deoxyctydine, G = 1.5) and 12.4 (cytosine, no G-value, determinable with optical detection). Ammonia, which is a product measured after γ-radiolysis of all the three compounds investigated (G(NH₃) = 0.6 ± 0.1), is not released within 15 ms after the pulse.

Introduction

Radiation induced changes in DNA have found wide interest and led to many investigations on its constituents, especially the nucleic acid bases [1]. Compared to the wealth of data on the radiolysis of the other pyrimidines, thymine and uracil, relatively little is known about the radiolysis of cytosine. Product analysis studies on the γ-radiolysis of cytosine were restricted to oxygenated solutions [2-6]. Because of the broad signals the ESR investigations of the cytosine OH adducts were not as successful as those of other pyrimidines [7-12]. In the pulse radiolysis studies [13-18] only the optical absorption technique has been used. The present work combines optical and conductivity detection. The latter allows to ascertain an ionization of radicals and products and yields G-values. From a comparison between the results of cytosine, 5-methyl cytosine and 2'-deoxyctydine the preferred site of OH attack may be located in these compounds.


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Results

In aqueous solutions of cytosine and its derivatives (5 • 10⁻⁴ mol • l⁻¹) saturated with N₂O and irradiated with 1 μs electron pulses only OH radicals (~90%) and H atoms (~10%) will be the primary species reacting with the pyrimidines.

1) Cytosine

At pH 6.6 the absorption spectrum immediately after the end of the pulse has two maxima at 340 and 440 nm (Fig. 1). At both wavelengths the decay of the optical absorption is of second order. Assuming a G-value of 6 for the absorbing species a rate constant of 2k = (6.5 ± 0.5) • 10⁸ 1 • mol⁻¹ • s⁻¹ is calculated.
At pH 11 the absorption spectrum immediately after the pulse (spectrum A in Fig. 2) is identical with that at pH 6.6 (Fig. 1). The absorptions at 340 and 440 nm (A in Fig. 2) decay by first order kinetics to give spectrum B within 30 µs (λ_max ~ 390 nm). Spectrum B is identical with the spectrum obtained directly after the pulse at pH = 13 (Fig. 3). The shape of the absorption of spectrum B for both pH values changes with time in a similar way (see below).

Fig. 2. N₂O saturated aqueous solution of cytosine at pH 11. Absorption spectrum of transients at the end, 5 and 30 µs after a 3.2 krads pulse. (O. D. for 1 cm pathlength.)

Fig. 3. N₂O saturated aqueous solution of cytosine at pH 13. Absorption spectrum of transients 0 and 100 µs after a 3.2 krads pulse. (O. D. for 1 cm pathlength.)

In Fig. 4 the half-lives of the decay from spectrum A to B have been plotted as a function of pH. The half life increases inversely proportional to the OH⁻ concentration. In the inset in Fig. 4 the derived bimolecular rate constant is plotted as a function of pH. The mean value calculated is \( k = (1.4 \pm 0.1) \cdot 10^8 \cdot \text{mol}^{-1} \cdot \text{s}^{-1} \). This set of experiments was repeated using a conductivity detecting system. The inset in Fig. 5 shows an example of the conductivity change with time. During the pulse, excess conductivity is generated by the formation of H⁺ and OH⁻. This excess conductivity rapidly disappears. A further decrease of the conductivity below the initial value prior to the pulse is due to the replacement of OH⁻ by a radical anion. The half-life of this process is a function of pH (Fig. 4) and was found to be identical with that for the change from spectrum A to B. From these conductivity changes G-values were calculated from the data taken at the end of the first order process (after minimum, inset Fig. 5), using 180 and 30 cm² · Ω⁻¹ · equivalent⁻¹ for the equivalent conductivity for OH⁻ and the radical anion, respectively. In order to eliminate the contribution

Fig. 4. Half-life of the OH⁻ induced reaction with one of the OH radical adducts to cytosine as a function of pH. ○ Optical absorption followed at 335 and 440 nm, △ conductivity measurements.

Fig. 5. N₂O saturated aqueous solutions of cytosine: Curve I, G-value of the conductivity change, after completion of the first order decay (see text) as a function of pH. Curve II, G-value of the conductivity change after completion of radical radical processes (15 ms after the pulse) as a function of pH.
due to the second order rise in conductivity (Fig. 5), the G-values corresponding to the minimum were measured as a function of dose rate and extrapolated to zero dose rate. A maximum G-value of 3.2 ± 0.3 has been found and an inflection point at pH 10.3 ± 0.1. At pH 11.1 this G-value is independent of the cytosine concentration (5 · 10⁻⁸–10⁻³ mol · l⁻¹) showing that no buffer effect of the substrate (pK cytosine = 12.2 [16]) simulates the obtained plateau. The spectrum B (Figs. 2 and 3) and the conductivity (Fig. 5) change again after the first order process just mentioned is completed. The conductivity increases by second order kinetics (2k = (6 ± 1) · 10⁻¹¹ · mol⁻¹ · s⁻¹).

A similar value (2k = (7 ± 1) · 10⁻¹¹ · mol⁻¹ · s⁻¹) was calculated from the increase in absorption at 335 nm with time. This value is independent of pH from 10–12.7. In contrast to the conductivity experiments a first order process (k < 2 · 10⁻³ s⁻¹) appears to be underlying the second order decay of the radicals.

In curve II of Fig. 5 the conductivity change 15 ms after the pulse, i.e. after completion of the radical reactions, is plotted as a function of pH. Inflection points are observed at pH 8.3 ± 0.2 and 10.7 ± 0.1. These inflection points are assigned to pK-values of products the G-values of which are 0.6 ± 0.1 (k(deprotonation) = (4.0 ± 0.2) · 10⁻¹ s⁻¹ measured in the pH range 8.3 to 9.3) and 1.5 ± 0.2. Products with very similar pK-values cannot be distinguished by such measurements and one cannot conclude, therefore, that only two products give rise to the observed effects. It is noted that ammonia (G(NH₃) = 0.5 ± 0.1; ⁶⁰Co γ-irradiation) is not generated within 15 ms after the pulse since ammonia would give rise to an inflection point at pH 9.3.

At higher pH values, out of the range of the conductivity set-up, a further pK-value (12.4 ± 0.2) of a product was determined by monitoring at λ = 310 nm 1 ms after the pulse, the minimal time required for completion of radical radical reactions. However at this high pH some products are not stable and undergo further reactions as indicated by the slow (t₁/₂ > 10 ms) decrease in absorption (300–350 nm).

2) 5-Methyl cytosine

The spectra at pH 7 (Fig. 6), pH 10.6 (Fig. 7) and pH 14.2 (Fig. 8) of the radicals obtained by the reactions of OH radicals with 5-methyl cytosine differ from those of cytosine (Figs 1–3).
At pH = 10.6, the decay kinetics of the intermediates as measured optically at 335, 440 and 500 nm and by conductivity change are very similar to the process observed with cytosine. After the fast OH-induced reaction (k = (1.4 ± 0.3) · 10^8 1 · mol\(^{-1}\) · s\(^{-1}\)) the resulting spectrum shows maxima at 390 and 550 nm. This absorption decays by second order kinetics (2k = (5.5 ± 0.5) · 10^8 1 · mol\(^{-1}\) · s\(^{-1}\)).

The G-value calculated from the conductivity change at pH 11.2 after completion of the fast process (Fig. 9) is only half (G = 1.6 ± 0.2) of that found in the cytosine system. The pK value appears to be shifted somewhat to higher pH, approx. 0.2 units. The conducting species, after completion of the radical reactions, has a pK-value of 10.6 ± 0.2 (Fig. 9). The G-value of the 5-methyl cytosine product (pK = 10.6) is about half (G = 0.8 ± 0.1) of that of the cytosine product (pK = 10.7). The slow first order process leading to the product observed with cytosine (pK = 8.3) is not observed. As in the case of cytosine, no ammonia is released within 15 ms.

At pH 10.5, immediately after the pulse, the absorption spectrum of the radical produced by the reaction of OH radicals with 2'-deoxycytidine and with cytosine are identical with respect to the positions of the maxima (Fig. 2). Compared with the spectrum obtained from cytosine the absorption at 335 nm is reduced by about 20–25% and enhanced by about 10–15% at 440 nm. No OH-induced reaction was observed in the pH-range 9.5 to 11.2 (optical and conductivity measurements). The slow first order process observed with cytosine (pK = 8.3) was also absent. The decay of the radicals as measured from optical absorption and the formation of product as measured from conductivity change is of second order (2k = (5.3 ± 0.5) · 10^8 1 · mol\(^{-1}\) · s\(^{-1}\)). The G-value of the conducting species (pK = 10.5 ± 0.2) formed during the decay is identical with the G-value of the product (pK = 10.7) obtained from cytosine (G = 1.5 ± 0.2). As in the case of cytosine and 5-methyl cytosine no ammonia is formed within 15 ms.

In the pH range from 11.3 to 13.7 (out of the range of the conductivity set up), immediately after the pulse, the optical density at 440 nm decreased by 35%, the inflection point being at pH 12.4 ± 0.1. This pK-value is likely to be a "kinetic pK-value" due to a competition of ‘OH and O" with 2'-deoxycytidine since the O" radicals react about three times slower with 2'-deoxycytidine than the OH radicals (Table I). An increase in optical absorption at 310 nm was observed following first order kinetics (k = (2.0 ± 0.2) · 10^4 s\(^{-1}\)). The rate was independent of [OH\(-\)] and of the substrate concentration (6 · 10\(^{-5}\)–10\(^{-3}\) mol · l\(^{-1}\)) thus indicating a monomolecular reaction. The resulting radical intermediates showed a pK-value of 12.7 ± 0.1. The radicals decayed by second order kinetics (2k = (5.5 ± 0.5) · 10^8 1 · mol\(^{-1}\) · s\(^{-1}\)).

Fig. 9. N\(_2\)O saturated aqueous solutions of cytosine: Curve I, G-value of the conductivity change after completion of the first order decay (see text) as a function of pH. Curve II, G-value of the conductivity change after completion of the radical radical processes (15 ms after the pulse) as a function of pH.
Table I. Data on rate constants, G-values and pK-values of radicals and products in the radiolysis of N$_2$O saturated aqueous solutions of cytosine, 5-methyl cytosine, and 2'-deoxyctydine as substrates (S).

<table>
<thead>
<tr>
<th>No.</th>
<th>Cytosine</th>
<th>5-Methyl cytosine</th>
<th>2'-Deoxyctydine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>k(OH + S) (\cdot 10^{-9} ) [l (\cdot) mol(^{-1}) (\cdot) s(^{-1})]</td>
<td>(6.3 \pm 1.5^a)</td>
<td>(6.0 \pm 1.5^a)</td>
</tr>
<tr>
<td>2</td>
<td>k(O(^-) + S) (\cdot 10^{-9} ) [l (\cdot) mol(^{-1}) (\cdot) s(^{-1})]</td>
<td>(1.8 \pm 0.3^{b,c})</td>
<td>(3.0 \pm 0.5^{c,d})</td>
</tr>
<tr>
<td>3</td>
<td>2k (radicals) (\cdot 10^{-8} ) [l (\cdot) mol(^{-1}) (\cdot) s(^{-1})]</td>
<td>(6.5 \pm 1)</td>
<td>(5.8 \pm 0.5)</td>
</tr>
<tr>
<td>4</td>
<td>k(OH(^-) + OH-adduct) (\cdot 10^{-8} ) [l (\cdot) mol(^{-1}) (\cdot) s(^{-1})]</td>
<td>(1.4 \pm 0.1)</td>
<td>(1.4 \pm 0.3)</td>
</tr>
<tr>
<td>5</td>
<td>G-values (pK-values) of species undergoing OH(^-)-induced reaction (No. 4)</td>
<td>(3.2) (10.3)</td>
<td>(1.6) (10.5)</td>
</tr>
<tr>
<td>6</td>
<td>Further pK-values of primary and secondary radicals</td>
<td>not observed</td>
<td>12.8</td>
</tr>
<tr>
<td>7</td>
<td>G-values (pK-values) of products</td>
<td>(0.6) (8.3)</td>
<td>absent</td>
</tr>
<tr>
<td>8</td>
<td>NH$_3$ formation 15 ms after the pulse</td>
<td>(0.5 \pm 0.1)</td>
<td>(0.7 \pm 0.1)</td>
</tr>
</tbody>
</table>

a From first order build-up of absorption (330–450 nm) at pH 7 and at \(5 \cdot 10^{-5}\)–8 \(\cdot\) 10\(^{-5}\) mol \(\cdot\) l\(^{-1}\) substrate concentrations.
b From first order build-up of absorption (cytosine 380–400 nm; 2'-deoxyctydine: 380–550 nm) at pH 13.2 and at \(5 \cdot 10^{-5}\)–10\(^{-4}\) mol \(\cdot\) l\(^{-1}\) substrate concentrations.
c Reaction of O\(^-\) with the pyrimidine monoanion. d See text.

The most relevant data are compiled in Table I. Included are data on the rate constants of OH radicals and O\(^-\) radicals with the substrates not explicitly mentioned in the text.

Discussion

In the radiolysis of water OH radicals, solvated electrons (e\(\text{aq}\)) and H atoms are the primary free radical species. In the presence of N$_2$O \((2.2 \cdot 10^{-2} \) mol \(\cdot\) l\(^{-1}\)) the solvated electrons are converted into OH radicals and at a pyrimidine concentration of \(5 \cdot 10^{-4}\) mol \(\cdot\) l\(^{-1}\) less than 5% of the solvated electrons react with the pyrimidine compounds (cf. k(e\(\text{aq}\) + N$_2$O) \[17\] and k(e\(\text{aq}\) + cytosine) \[18\]). The OH radicals (G = 5.4) and the H atoms (G = 0.6) are the primary radicals reacting with the pyrimidines. The pK-value of the OH radical is 11.9 \[19\] so that, at much higher pH, the O\(^-\) radical is expected to be the reacting species.

The absorption spectra of the OH adduct radicals of cytosine \[13–15\] and 5-methyl cytosine \[14\] have already been reported as well as the rate constants of the bimolecular decay of these radicals \[13–15\] at neutral pH. The present results (absorption spectra and bimolecular rate constants) are in agreement with those from the literature \[13–15\]. The reaction of a cytosine OH-adduct radical with OH\(^-\) has also been reported \[13, 14\]. From the data presented the following conclusions can be drawn.

1) Rate constants of the reactions of OH and O\(^-\) radicals with cytosine and its derivatives

The rate constants for the reactions of the OH radicals with cytosine, 5-methyl cytosine and 2'-deoxyctydine are around \(6 \cdot 10^9\) \(\cdot\) l \(\cdot\) mol\(^{-1}\) \(\cdot\) s\(^{-1}\) (Table I) (cf. k(cytosine + OH) = 4.9 \(\cdot\) 10\(^9\) \(\cdot\) l \(\cdot\) mol\(^{-1}\) \(\cdot\) s\(^{-1}\) \[20\]). The rate constants for the reactions of the O\(^-\) radicals with these compounds are not much lower \((1.2 \cdot 10^9 — 3 \cdot 10^9\) \(\cdot\) l \(\cdot\) mol\(^{-1}\) \(\cdot\) s\(^{-1}\) \[21\]). These rate constants appear to be very high since O\(^-\) has been shown to add three orders of magnitude slower to aromatic compounds than the OH radical \[21\]. The rate constants for hydrogen abstraction by O\(^-\) \((k = 10^9\) \(\cdot\) l \(\cdot\) mol\(^{-1}\) \(\cdot\) s\(^{-1}\) \[21\] (e.g. from alcohols) are only reduced by a factor of about 2 as compared to those of OH radicals.

In cytosine and its derivatives hydrogen abstraction might occur at the amino group in cytosine, at the amino and methyl groups in 5-methyl cytosine, and at the amino group and the sugar moiety in 2'-deoxyctydine. The similarities of the absorption spectra at pH 7 and 13 obtained with 2'-deoxyctydine, however, suggest that O\(^-\) adds also to the pyrimidine ring. The 35% decrease of the intensity of the 440 nm absorption band ("pK" = 12.4) may be due to a different site of attack at the pyrimidine ring and/or due to a more pronounced hydrogen abstraction from the sugar moiety by the O\(^-\) radical in comparison to the OH.
The shift of the pK-value of \( \text{OH}^- \) (11.9) to the observed "pK-value" of 12.4 is explained by the ratio of \( k(\text{OH}^- + 2'-\text{deoxycytidine}) \) over \( k(\text{OH}^- + 2'-\text{deoxycytidine}) \) which equals 3 (Table I).

2) Evaluation of radical attack at C-5 and C-6

It is generally accepted that the major reaction of the OH radicals with the pyrimidine bases is their addition to the C-5-C-6 double bond \([1, 22]\). This reaction would give rise to the radicals 1 and 2. In contrast to the other pyridimidine bases cytosine has a double bond between N-3 and C-4. OH addition at this position might result in radical 3. In 5-methyl cytosine the allyl radical 4 might be formed by hydrogen atom abstraction from the methyl substituent at C-5 \([10, 23]\).

For cytosine and its derivatives the relative attack at C-5 and C-6 is not known. ESR studies \([7-12]\) on radicals derived from the reaction of OH radicals with pyrimidines indicate that the preferred OH attack is at C-5 but that a methyl substituent at C-5 favours attack at C-6 \([7]\). This has been thought to be due to the electrophilic nature of the OH radical \([24]\). A substituent at N-1 may not have such a strong directive effect as one at C-5.

As shown in Figs. 5 and 9 only a fraction of the primary adduct radicals in cytosine (Fig. 5) and 5-methyl cytosine (Fig. 9) are capable of an OH-induced reaction \([k = 1.4 \times 10^9 \text{ mol}^{-1} \text{ s}^{-1}]\). The G-value of this process has been found to be 3.2 for the cytosine and 1.6 for the 5-methyl cytosine adduct radicals. Apparently only one of the radicals 1 and 2 shows this reaction. The effective attack of the OH radical on the primary adduct radicals in cytosine (Fig. 5) and 5-methyl cytosine (Fig. 9) is known. For 5-methyl cytosine a pK value of 10.4 has been observed by ESR for a number of pyrimidines \([10]\).

A rearrangement of the C-6 OH adduct radical (reaction (2)) has been observed by optical detection with glycine anhydride and show the expected diffusion controlled reaction with the hydroxide ion \([26]\). Thus the reaction might be an OH-induced rearrangement of radical 1 leading to a radical which can deprotonate. Similar processes appear to have been observed with other pyrimidines \([15]\). In our case the hydroxide ion has been shown to be a good nucleophile and is capable of attacking radical 1 at the methyl group. This reaction is not yet understood and warrants further investigation.

Radicals which are similar to 1 have been observed by ESR for a number of pyrimidines \([10]\).

In the preceding section it has been shown that the OH-adduct radicals show a similar behaviour as the OH-adduct radicals of cytosine and 5-methyl cytosine. Assuming that the OH-adduct radicals show a deprotonation reaction \([25]\) as given by reaction (1).

In addition at this position result in radical 3.

The G-value of this reaction has been found to be about 27% in the case of 5-methyl cytosine compared to cytosine. Therefore a hydrogen at N-1 is necessary for this reaction to be detected. The mechanism of this reaction is not yet understood and warrants further investigation.

3) Reactions of the adduct radicals

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If this rearrangement occurs within 1–2 μs, we would not be able to observe it under our experimental conditions. The rearranged radical 5 has no acidic hydrogen atoms and no conductivity change induced by its dissociation would be expected in the pH range accessible to the conductivity set-up (up to pH 11.3). At present it is not possible to distinguish between radicals 2 and 5.

The first order process \( (k = 2 \cdot 10^3 s^{-1}) \) observed with a 2-deoxyctydine adduct radical is independent of pH. The resulting O−− adduct radical showed a pK-value of 12.7.

Independent of [OH−] a first order process \( (k = 2 \cdot 10^4 s^{-1}) \) was underlying the bimolecular decay of the cytosine adduct radicals. No reaction of this type was monitored in the case of 5-methyl cytosine. With 5-methyl cytosine an O−− adduct radical was observed immediately after the pulse (Figs 6–8) with a pK-value of 12.8. None of these reactions and pK-values could be assigned to well defined radicals.

c) pK-Values of products and ammonia formation

The pK-values of some products were observed after completion of the bimolecular decay of the radicals (Table I) but cannot be connected with known products as no product analysis is available yet. In γ-radiolysis ammonia is released from cytosine and its derivatives with a G-value of about 0.6. Ammonia formation appears to be slow compared to the free radical processes and is not significant 15 ms after the pulse (cf. Figs 5 and 9). Therefore ammonia can only be released from a product (or products). This product might have radical 3 as precursor. Radical 3 has a labile hemi-aminal structure.

**Experimental**

Cytosine (Merck), 2′-deoxyctydine (Bohringer) and 5-methyl cytosine (Serva) were used without further purification and dissolved in triply distilled water. The pH was adjusted with NaOH. In general \( 5 \cdot 10^{-4} \text{ mol} \cdot \text{l}^{-1} \) solutions of substrate saturated with \( N_2O \) (Hoechst) which had been freed from residual oxygen by an Oxsorb column (Messer Griesheim) were irradiated with 1 μs electron pulses (0.3–4 krads) from a van de Graaff accelerator as described previously [27, 28]. Because of the initial conductance due to \( Na^+ \) and \( OH^- \) ions, conductivity measurements were limited up to pH 11.3. Ammonia was measured after exposure of the above solutions to 60-Co-γ rays (dose rate \( 3 \cdot 10^{16} \text{ eV} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \) in the dose range from \( 0.75 \cdot 10^{18} \) to \( 1.5 \cdot 10^{18} \text{ eV} \cdot \text{g}^{-1} \) and in the pH range 1–12 using an ammonia sensitive electrode (Orion).

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