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 [13-15] 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'-deoxycytidine the preferred site of OH attack may be located in these compounds.

Results

In aqueous solutions of cytosine and its derivatives (5 • 10⁻¹⁴ mol⁻¹ s⁻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) • 10⁻¹¹ s⁻¹ is calculated.

Fig. 1. N₂O saturated aqueous solution of cytosine at pH 6.6. Absorption spectrum of transients 0 and 100 µs after a 3.2 krads pulse. (O. D. for 1 cm pathlength.)
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 ($\lambda_{\text{max}} \sim 390$ 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. N$_2$O 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.)](image1)

![Fig. 3. N$_2$O 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.)](image2)

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 $k = (1.4 \pm 0.1) \cdot 10^8$ mol$^{-1}$ 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 cm$^2$ 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.](image3)

![Fig. 5. 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 radical radical processes (15 ms after the pulse) as a function of pH.](image4)
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 $3.2 \pm 0.3$ has been found and an inflection point at pH $10.3 \pm 0.1$. At pH $11.1$ this G-value is independent of the cytosine concentration ($5 \cdot 10^{-3}$–$10^{-3}$ mol $\cdot$ 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 ($2k = (6 \pm 1) \cdot 10^{8} \cdot$ mol$^{-1}$ $\cdot$ s$^{-1}$).

A similar value ($2k = (7 \pm 1) \cdot 10^{8} \cdot$ mol$^{-1}$ $\cdot$ 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 ($k \approx 2 \cdot 10^{3}$ 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 $8.3 \pm 0.2$ and $10.7 \pm 0.1$. These inflection points are assigned to pK-values of products the G-values of which are $0.6 \pm 0.1$ (k(deprotonation) = $(4.0 \pm 0.2) \cdot 10^{8}$ s$^{-1}$ measured in the pH range 8.3 to 9.3) and $1.5 \pm 0.2$. 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(NH$_3$) = $0.5 \pm 0.1$; $^{60}$Co 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 ($12.4 \pm 0.2$) of a product was determined by monitoring at $\lambda = 310$ 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 ($t_{1/2} > 10$ 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).

![Fig. 6. N$_2$O saturated aqueous solution of 5-methylcytosine at pH 7. Absorption spectrum of transients 0 and 50 µs after a 2.2 krad pulse. (O. D. for 1 cm pathlength.)](image6.png)

![Fig. 7. N$_2$O saturated aqueous solution of 5-methylcytosine at pH 10.6. Absorption spectrum of transients 0 and 50 µs after a 3.2 krad pulse. (O. D. for 1 cm pathlength.)](image7.png)

![Fig. 8. N$_2$O saturated aqueous solution of 5-methylcytosine at pH 14.2. Absorption spectrum of transients 0 and 50 µs after a 2.2 krad pulse. (O. D. for 1 cm pathlength.)](image8.png)
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 = \(1.4 \pm 0.3\) \( \times 10^8\) \( \text{mol}^{-1} \cdot \text{s}^{-1}\)) the resulting spectrum shows maxima at 390 and 550 nm. This absorption decays by second order kinetics (2\(k = (5.5 \pm 0.5) \times 10^8\) \( \text{mol}^{-1} \cdot \text{s}^{-1}\)).

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.

![Fig. 9. N\textsubscript{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.](image)

At pH 14.2 (Fig. 7) the spectrum immediately after the pulse shows absorption maxima at 370 and 530 nm. This spectrum is assigned to an intermediate (intermediates) with a pK-value of 12.8 ± 0.1 as determined at \(\lambda = 350, 370\) and 530 nm immediately after the pulse. This pK-value is not a "kinetic pK-value" due to a competition of ‘OH and O‘ (pK = 11.9) with 5-methyl cytosine since the rate of reaction of ‘OH and O‘ with 5-methyl cytosine are quite similar. This has been shown by measuring at low substrate concentration (6 \(\times 10^{-5}\)\( \text{mol} \cdot \text{L}^{-1}\)) the build-up of the absorbance at 370 and 530 nm (pH = 13.7 and 1 krad pulse) which is of first order (\(t_{1/2} = 4\ \mu\text{s}\)) and corresponds to a rate constant of \(k = 3 \times 10^9\) \(\text{mol}^{-1} \cdot \text{s}^{-1}\) for the reaction of O- with the 5-methyl cytosine anion (pK = 12.4 [16]). At this low substrate concentration the equilibrium of O- and ‘OH is established before appreciable reaction of these species with the substrate occurs. The substrate radical decays by second order kinetics (\(2k = (5.8 \pm 0.5) \times 10^8\) \(\text{mol}^{-1} \cdot \text{s}^{-1}\)) in the pH range from 10.5 to 13.8.

3) 2'-Deoxycytidine

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 (2\(k = (5.3 \pm 0.5) \times 10^8\) \(\text{mol}^{-1} \cdot \text{s}^{-1}\)). 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) \(\times 10^4\)\( \text{s}^{-1}\)). The rate was independent of [OH-] and of the substrate concentration (6 \(\times 10^{-5}\)–10\(^{-3}\) \(\text{mol} \cdot \text{L}^{-1}\)) 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 (2\(k = (5.5 \pm 0.5) \times 10^8\) \(\text{mol}^{-1} \cdot \text{s}^{-1}\)).
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−aq) and H atoms are the primary free radical species. In the presence of N2O (2.2 • 10−2 mol • l−1) the solvated electrons are converted into OH radicals and at a pyrimidine concentration of 5 • 10−4 mol • l−1 less than 5% of the solvated electrons react with the pyrimidine compounds (cf. k(e−aq + N2O) [17] and k (e−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′-deoxycytidine are around 6 • 108 l • mol−1 • s−1 (Table I) (cf. k(cytosine + OH) = 4.9 • 109 l • mol−1 • s−1 [20]). The rate constants for the reactions of the O− radicals with these compounds are not much lower (1.2 • 109 — 3 • 109 l • mol−1 • s−1, Table I). 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 = 108 l • mol−1 • 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′-deoxycytidine. The similarities of the absorption spectra at pH 7 and 13 obtained with 2′-deoxycytidine, 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
radical. The shift of the pK-value of $\cdot OH/O^-$ (11.9) to the observed "pK-value" of 12.4 is explained by the ratio of $k(OH + 2'-deoxycytidine)$ over $k(O^- + 2'-deoxycytidine)$ 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 pyrimidine 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 ($k = 1.4 \cdot 10^8 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$) giving rise to radicals which have a pK value of 10.4. 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 directive effect of the methyl substitution at C-5 disfavours the OH addition at C-5. From the lower yield of a radical with a pK of around 10.4 in the case of 5-methyl cytosine compared to cytosine it is proposed that this species is most likely the C-5 adduct radical (1). Assuming that the H-adduct radicals show a similar behaviour as the OH-adduct radicals it is estimated that about 55% of all radicals (G(H + OH) = 6) add to C-5 in the case of cytosine and only about 27% in the case of 5-methyl cytosine.

3) Reactions of the adduct radicals

a) OH- induced reaction of the C-5 adduct radical of cytosine and 5-methyl cytosine

In the proceeding section it has been shown that the OH- induced conductivity change (Figs 5 and 11) might be due to the adduct radical at C-5, radical 1. The rate of this reaction is $1.4 \cdot 10^8 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$. This rate constant appears to be too low by two orders of magnitude for a deprotonation reaction [25] as given by reaction (1).

$$R^1: H \text{ or } CH_3$$

Radicals which are similar to 1 have 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 this reaction has only been seen with cytosine and 5-methyl cytosine but not with 2'-deoxycytidine. 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.

b) Rearrangements and pK-values of other adduct radicals

A rearrangement of the C-6 OH adduct radical (reaction (2)) has been observed by ESR for a number of pyrimidines [10].
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 \([\text{OH}^-]\) a first order process \( (k = 2 \cdot 10^3 s^{-1}) \) 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.

e) 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 \( \gamma \)-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 N2O (Hoechst) which had been freed from residual oxygen by an Oxysorb column (Messer Griesheim) were irradiated with 1 μs electron pulses \((0.3–4 \text{ krads})\) 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-\( \gamma \) rays (dose rate \( 3 \cdot 10^{10} \text{ eV} \cdot \text{g}^{-1} \cdot \text{h}^{-1} \)) 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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