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MECHANISMS FOR RADIATION DAMAGE IN
DNA CONSTITUENTS AND DNA: REACTIONS OF
THE N_1 -SUBSTITUTED THYMINE π -CATION RADICALS¹

MASTER

(Short Title: Thymine π -Cation Radical Reactions)

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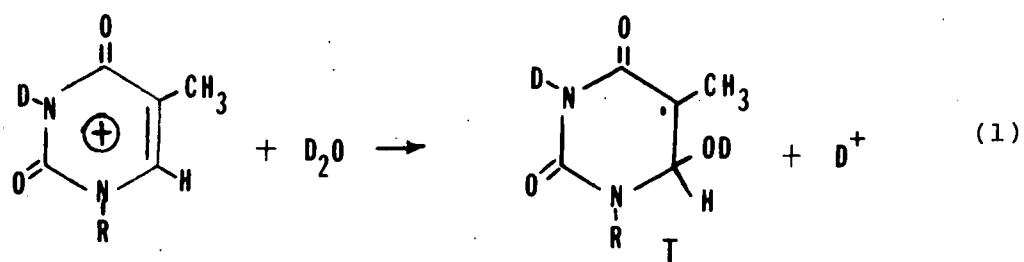
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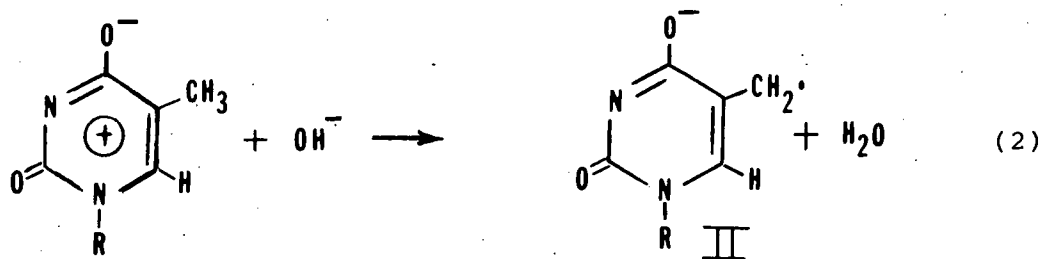
ABSTRACT

Reactions of the N_1 -substituted thymine π -cation radicals were investigated by e.s.r. in several aqueous glasses. In 8M NaOD (NaOH) spectra suggestive of $OD^-(OH^-)$ addition to position 6 in the π -cations of 1-methylthymine, and thymidine were found immediately after u.v. photolysis at 77K. Production of the same radicals by electron attachment to 1-methyl-5-bromo-6-hydroxythymine, and 5-bromo-6-hydroxythymidine in 8M NaOD confirms the OD^- addition mechanism. Results found for these brominated compounds in 12M LiCl(D₂O) after electron attachment show that the $a_6(H)$ splitting was sensitive to changes in substituents at position 1 as well as changes in environment. This variation in splitting is shown to be accounted for by small conformational changes in the radicals. In 8M NaClO₄ π -cations of the substituted thymines gave evidence for both deprotonation and OD^- addition.

Recent e.s.r. investigations have proposed a number of radiation mechanisms in DNA. Results with γ -irradiated DNA at low temperatures have indicated that anion radicals are formed predominantly on thymine and the cation is formed on guanine.² Other work by Gregoli, Olast and Bertinchamps has suggested that the positive ions of purine and pyrimidine nucleotides react by OH^- addition to form a neutral radical.³ Reaction 1 shows this mechanism for thymidine ($\text{R}=\text{deoxyribose}$) in D_2O .



In previous publications from this laboratory we reported a mechanism for the reaction of the cation radicals of a number of 5-methylpyrimidine DNA bases in several aqueous glasses.^{4,5} Reaction 2 shows this mechanism in a basic medium.



In this work we report evidence for reactions 1 and 2 occurring in N_1 -substituted thymine π -cations. The relative rate of reactions 1 and 2 is found to be dependent on the substituent at position 1 and the concentration of base.

EXPERIMENTAL

The DNA components used in this study were obtained from Sigma with the exception of 1-methylthymine which was obtained from Cyclo Chemical Company. Brominated compounds were prepared by reaction of the DNA base with bromine water.^{6,7} When possible the products were

recrystallized. In each case n.m.r. gave evidence for a virtually complete reaction.

The experimental apparatus and procedures employed in this study have been detailed in our previous investigations.^{4,5,8,9,10} Two techniques have been employed in this study to generate radicals. They are photoionization in 8M NaOD or 8M NaClO₄(D₂O) glasses⁸ or electron attachment in 8M NaOD or 12M LiCl(D₂O) glasses.⁹ In the latter technique the solution 8M NaOD or 12M LiCl containing $2 \times 10^{-2} M$ K₄Fe(CN)₆ and ca. 1 mM solute is cooled to 77K. U.v. photolysis (254 nm) of the K₄Fe(CN)₆ generates electrons which react with the solute or are trapped in the glass. The trapped electrons are made mobile by photobleaching with an incandescent lamp.

In the experiments reported in this paper deuterated glasses were employed for improved resolution. Except for differences in resolution protonated glasses gave analogous results.

Due to the instability of the 5-bromo-6-hydroxythymine derivatives in basic solution, these compounds were added to previously cooled (-20°C) solutions.

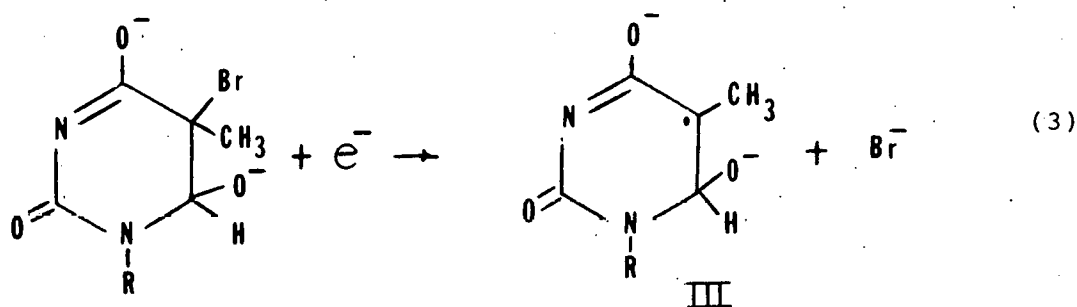
Freymy's salt was employed as a standard for g values and hyperfine splittings ($a = 13.1$, $g = 2.0056$).

Results and Discussion

I. Reactions of π -Cations in a Alkaline Glass (8M NaOD)

In Figure 1A we show the result of photoionization of 1-methylthymine (1-Met) with 254 nm light in 8M NaOD at 77K in the presence of an electron scavenger (1-2 mM K₃Fe(CN)₆). This e.s.r. spectrum contains

a small amount of anion which adds a 16G doublet to the central portion of the spectrum.¹⁰ In Figure 1B the sample has been warmed to 190 K and cooled to 100 K. The difference in Figures 1A and 1B is due to removal of the anion and a change in one coupling which results in an improvement in resolution upon warming. Analyses of these spectra yield the values reported in Table 1. Figure 1C shows the result of electron attachment to 1-methyl-5-bromo-6-hydroxythymine (MeBrOht) in 8M NaOD at 77 K, followed by warming to 190 K and cooling to 110 K. Electrons are generated by the photooxidation of $K_4Fe(CN)_6$. The radical (III R=CH₃) suggested to be produced by electron attachment is shown in reaction 3.



Analysis of the spectrum in Figure 1C yields identical parameters within experimental error as found from the analysis of Figure 1B (see Table 1). The assignments in Table 1 of the large splitting due to three protons to the 5-methyl group and the smaller splitting due to a single proton to the methylene-like proton at position 6 are the only reasonable assignments considering the structure of this radical.

The above results are considered excellent evidence that the 1-Met π -cation undergoes a reaction similar to reaction 1, perhaps by reaction with OD^- instead of D_2O , to form a radical which subsequently dedeuterates from the OD group to form radical III. The results of Neta with

similar radicals clearly suggests that the OH group in radical III would be in its deprotonated form in an alkaline medium.¹¹

The only other possible radical which could account for the spectra in Figures 1A and 1B is the π -cation radical. However this species has been observed in basic 8M NaClO₄ glasses (see ref. 8 and Figure 5D below) and it has considerably different couplings and g values. Thus the results found here for 1-Met in 8M NaOD suggest that the π -cation is not stable even at 77 K.

Results found previously for thymidine (T) (ref. 12 Figure 5) and in this work for 5-bromo-6-hydroxythymidine (BrOHT) (Figures 2A and 2B) in 8M NaOD are analogous to those found for 1-Met and MeBrOHT. The hyperfine splittings for the T π -cation reaction product in 8M NaOD and those found for the radical produced by electron attachment to BrOHT in 8M NaOD are found to be virtually identical after warming to 190K (see Table 1).

In an earlier report¹² we suggested that the T π -cation was stabilized at 77 K in 8M NaOD. However the g values reported did not correspond to the g values of other thymine π -cations and the stability of the radical was not in accord with other 5-methylpyrimidine π cations. There was therefore an uncertainty to this identification. We believe the reinterpretation presented in this work clearly shows that the T π -cation is not stable in 8M NaOD and reacts by OD⁻ addition at position 6 to form radical III with R=deoxyribose.

II. Brominated Compounds in a Neutral Glass (12M LiCl)

In Figures 3A and 4A we show the result of electron attachment to MeBrOHT and BrOHT at 77 K in 12M LiCl(D₂O). Upon warming samples

to temperatures where the glass has softened and recooling to 105 K the spectra in Figures 3B and 4B are found. The analysis of these spectra is given in Table 1. As can be seen it is the $a_6(H)$ splitting which is altered most on warming. In the case of MeBrOht it is sufficiently increased to make the spectrum appear as a quintet due to 4 equivalent protons. Although through computer simulations the value of $a_6(H)$ was found to be somewhat less than $a_5(CH_3)$. Others have reported radicals of this type (I) after OH \cdot attack on thymidine and thymine in aqueous solution.^{13,14} The values reported are also listed in Table 1. They are quite close to those found here for the warmed samples.

In the 12M LiCl matrix the radicals produced by debromination should be of the form of radical I. The differences in structure between radicals I and III alters the hyperfine splittings somewhat at the low temperatures but has a more pronounced effect after the warming step. As can be seen in the Table, the major difference caused by the structural changes is a difference in the value of $a_6(H)$. This splitting appears to be highly sensitive to changes in molecular structure and environment. The variation in splitting observed for these radicals is easily explained by the dependence of β -proton splittings on its orientation (θ) to the p orbital containing the unpaired electron.¹⁵ In this case there are two sites, positions 1 and 5, which contribute to the coupling. The major coupling is obviously to position 5 ($\rho_5^\pi = 0.8$ assuming $Q(CH_3) = 29$). One can employ effective $B\rho^\pi$ in the relation

$$a = B\rho^\pi \cos^2 \theta \quad (1)$$

to account for the contribution from positions 1 and 5. In other work on the very similar radicals a value near 60G was indicated for $B\rho_{\text{eff}}^{\pi}$.¹⁶ Employing this value and relation (1) we find 6 to 20G splittings place θ between 72° and 55° . Thus only a moderate change in orientation results in a relatively large change in the value of $a_6(\text{H})$.

The change noted in $a_6(\text{H})$ on warming is found in all the radicals in Table 1 and is due to a shift to a more stable molecular conformation upon the softening of the various glasses. It should be noted that all the values found for $a_6(\text{H})$ suggest that the OD group is at an orientation so that the oxygen points up from the plane of the ring (θ near 0°).

III. Reactions of π -Cations of TMP and 1-Methylthymine in 8M $\text{NaClO}_4(\text{D}_2\text{O})$

Photoionization of thymidine-5'-monophosphate (TMP) or 1-methylthymine (1-Met) in 8M $\text{NaClO}_4(\text{D}_2\text{O})$ containing $5 \times 10^{-2}\text{M}$ NaOD at 77 K produces the e.s.r. spectra of the π -cations (Figures 5A & D respectively). These spectra have been fully interpreted in our previous work⁸ and each was shown to be an overlap of the π -cation of the parent compound and O^- produced by e^- reaction with ClO_4^- . Upon warming the samples to 180 K for a short period and recooling to 110 K the signal due to the cation and O^- are lost to give Figures 4B and 4E. We suggest there are two major radicals in these spectra. The first is radical II with $\text{R}=\text{CH}_3$ for 1-Met and $\text{R}=\text{deoxyribose phosphate}$ for TMP. This species has been identified as a decay product of the thymine π -cation in our previous work in 8M NaOD and 5M K_2CO_3 glasses. Since O^- has been shown to abstract from the 5-methyl group, the

appearance of this radical can simply be explained by O^- attack on the parent compound.^{11,17,18} However adding up to 1M $NaHCO_2$ which effectively scavenges O^- , only reduced but did not remove the signal due to radical II. In addition radical II has a characteristic absorption in the visible at 550nm,¹⁹ which imparts a pink color to the sample. The pink color was only reduced in intensity but not removed by addition of 1M $NaHCO_2$. The results suggest that this species is produced by reaction 2 in addition to O^- attack on the parent compound.

The two outer components in Figure 4B and 4E show the presence of another species. Warming 1-Met samples to ca. 190K results in the gradual loss of the signal due to radical II while the second species remains but does not appear to increase in intensity (Figure 4F). The five lines in Figure 4F are separated by an average of 21.1G. In view of the variability found for the $a_6(H)$ splitting (Table 1) it is not unreasonable to suggest a radical of the form of radical I. To further test this hypothesis electron attachment to MeBrOht was attempted in a basic 8M $NaClO_4$ glass. In this glass the ClO_4^- is an efficient electron scavenger therefore the concentration of MeBrOht was kept high ($2 \times 10^{-2}M$). Electron generation by photo-ionization of deoxyguanosine (dG) proved to be more successful than $K_4Fe(CN)_6$. Thus the spectra (Figure 6) are overlapped with a singlet due to the dG positive ion in the central portion of the spectrum. In Figure 6A and 6B the spectrum of radical III (R=Me) generated by the above technique is shown at 110K and at 175K, respectively. As can be seen upon warming the spectrum converts to a quintet whose

components are separated by 20.9G. This value is within experimental error of that found for l-Met. The spectrum found for the final radical of TMP (Figure 4C) was not reproduced by electron attack on the brominated compound in NaClO_4 . Some variability was found in the final spectrum for TMP. Thus the difference in spectra may be due to solely a conformation difference.

The results for l-Met are considered good evidence for reactions 1 and 2 both occurring in 8M NaClO_4 . The results for TMP may suggest another competing mechanism in this case. The only reasonable mechanism which could explain the 5 line spectra found for TMP would be one involving the attack of a radical species such as $\cdot\text{O}^-$, or radical II at C-6 in the parent compound. Experiments which varied the concentration or added $\cdot\text{O}^-$ scavengers did not give evidence for such a mechanism for l-Met. This combined with the fact that the bulkiness of the deoxyribose phosphate group in TMP would tend to hinder bimolecular reactions in the viscous glass suggests a final radical of the form of radical I for TMP as well.

It should be noted however that results found by Gregoli and Bertinchamps (discussed below) are suggestive of a bimolecular attack of radical II on TMP.

Conclusions

The results found in this work show the π -cations of N_1 -substituted thymine undergo certain decomposition reactions. In 8M NaOD or NaOH we find the cations of N-substituted thymine add OD^- or OH^- (reaction 1) whereas in previous work we found that thymine itself deprotonated (reaction 2) in this medium.⁵ In 8M NaClO_4 the substituted thymine appear to undergo both reactions.

Recent results found from pulse radiolysis studies of aromatic cation radicals have some bearing on this work.^{20,21} Holcman and Sehested have shown that the anisole cation reacts with OH^- to form a hydroxyl adduct.²⁰ This type of reaction was first suggested by Norman et al. for a number of cations of unsaturated hydrocarbons including benzene.²² The deprotonation reaction found in our work is also known to occur in hydrocarbon cations. For example Holcman and Sehested have recently shown that methylated benzene in aqueous solution undergo deprotonation resulting in benzyl radicals.

Thus the results observed in this work are those expected from studies of hydrocarbon cations. It is likely that the analogy to hydrocarbon cation reactions holds because the charge density in N_1 -substituted thymine is removed chiefly from the 5,6 double bond of the thymine base.

Gregoli and Bertinchamps have suggested that the TMP π cation produced in frozen aqueous solutions of TMP undergoes the same OH^- addition reaction as found here. However, the spectrum identified as the precursor to the hydroxyl adduct is suggestive of radical II not a π -cation.^{5,23} Since bimolecular attack of radical II at C-6 on TMP would produce a spectrum similar to the OH^- adduct this mechanism must be considered as a possible alternative explanation.

Table I. E.S.R. Parameters for N₁-Substituted-6-hydroxythymine radicals.

COMPOUND	RADICAL	MATRIX	METHOD	a ₅ (CH ₃)	a ₆ (H)	Temp (K)
1-Methylthymine	III(R=CH ₃)	8M NaOD	B	22.3 G 21.9	8.0 G 10.0	110 190
1-Methylthymine	III(R=CH ₃)	8M NaClO ₄ (Basic)	B	21.1	21.1	190
1-Methyl-5-bromo-6-hydroxythymine	III(R=CH ₃)	8M NaOD	A	21.3 22.1	9.0 10.0	110 190
1-Methyl-5-bromo-6-hydroxythymine	I (R=CH ₃)	12M LiCl	A	23.5 23.0	9.7 17.5	110 165
1-Methyl-5-bromo-6-hydroxythymine	III(R=CH ₃)	8M NaClO ₄ (Basic)	C	23.0 20.9	9.5 20.9	110 175
Thymidine	III(R=deoxyribose)	8M NaOD	B	21.7 21.6	6.0 8.0	110 190
5-Bromo-6-hydroxy-thymidine	III(R=deoxyribose)	8M NaOD	A	21.2 21.7	7.0 8.1	110 190
5-Bromo-6-hydroxy-thymidine	I (R=deoxyribose)	12M LiCl	A	23.7 23.6	6.5 12.1	110 185
TMP	III(R=deoxyribose phosphate)	8M NaClO ₄ (Basic)	B	21.7	21.7	195
5-Bromo-6-hydroxy-thymine	I (R=H)	12M LiCl	A	23.6 23.1	9.5 17.5	110 165
Thymidine	I (R=deoxyribose)	Aqueous solution	D	23.6	10.8	ambient
Thymine	I (R=H)	Aqueous solution	D	22.4 ±0.5	15.1 ±0.5	ambient

FOOTNOTES TO TABLE 1

All g values were 2.0033 ± 0.002 .

- A. Electron attachment to solute. Electrons are generated by the photoionization of $K_4Fe(CN)_6$.
- B. Reaction of the π -cation radical with OD^+ or D_2O as in reaction 1.
- C. Electron attachment to solute. Electrons are generated by the photoionization of deoxyguanosine.
- D. Hydroxyl attack on solute utilizing a flow system technique. See refs. 13 and 14.

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FIGURE LEGENDS

Figure 1A First derivative e.s.r. spectrum of radical III ($R = CH_3$) in 8 M NaOD produced by u.v. photoionization of 1-methylthymine (1-Met) at 77K in the presence of an electron scavenger (recorded at 110 K)

B Spectrum of sample in A after warming to 190 K and cooling to 110 K

C. Spectrum of radical III ($R=CH_3$) at 110 K (after warming to 190 K) produced by electron attachment to 1-methyl-5-bromo-6-hydroxythymine in 8 M NaOD. The agreement between 1B and 1C shows the π -cation of 1-Met undergoes OD^- addition (reaction 1)

Figure 2 E.S.R. spectra of radical III ($R=deoxyribose$) produced by electron addition at 77 K to 5-bromo-6-hydroxythymidine in 8 M NaOD.

A. recorded at 110 K B. after warming to 190 K and recooling to 110 K.

Figure 3 E.s.r. spectra of radical I ($R=CH_3$) produced by electron attachment at 77 K to 1-methyl-5-bromo-6-hydroxythymine in 12M LiCl (D_2O).

A. recorded at 110 K B. after warming to 165 K and recooling.

Figure 4 E.s.r. spectra of radical I ($R=H$) produced by electron attachment to 5-bromo-6-hydroxythymidine at 77 K in 12 M LiCl(D_2O).

A. recorded at 110 K B. recorded after warming to 165 K and recooling.

Figure 5 A(D) E.s.r. spectra of the π -cations of (A) (TMP) and (D) 1-methylthymine in basic 8 M $NaClO_4$. The broad low field signal is due to O^- . Recorded at 110 K.

B(E) Spectra of radicals II and III produced by warming the π -cations to 180 K and recording at 110 K. B. TMP, E. 1-Met

C(F) Spectra of radical III (and or another similar species for TMP) after further annealing at 190 K. C. TMP F. 1-Met (Gain Increased)

Figure 6 E.s.r. spectra produced by electron attachment to 1-methyl-5-bromo-6-hydroxythymine in basic 8 M $NaClO_4$ (D_2O).

A. Radical I ($R=CH_3$) at 110 K

B. Radical I ($R=CH_3$) at 175 K

