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MECHANISMS FOR RADIATION DAMAGE IN DNA CONSTITUENTS AND DNA

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The initial effects of all forms of ionizing radiation on DNA are the production of positive ions, negative ions and excited states of the DNA bases. These species may later react to produce biologically significant damage. Ion radicals in γ -irradiated DNA have been reported.¹⁻³ For example Gräslund, Ehrenberg, Rupprecht, and Ström suggest an anion radical on thymine and a cation radical on guanine in γ -irradiated oriented DNA at 77 K.¹ The reactions of these ion radicals produced individually in DNA bases have been recently investigated with some success.⁴⁻¹⁰ In this discussion we will concentrate on the initial events immediately after ionization i.e. the fate of the electron and positive hole in irradiated DNA.

There are a number of techniques which have been used to produce ion radicals in DNA bases for ESR studies. They include radiolysis, electrolysis and photolysis. For the most part our studies have employed photolytic techniques. Below we describe these techniques and several recent studies on the ion radicals of DNA constituents.

A. The formation of DNA Base π -Cation* Radicals by Photoionization.

In early work by Helens, Santus, and Douzou the cation radicals of several purines were reported to be produced through

*The terms π -cation and π -anion refer to the loss or gain of one electron from the π electron system and do not refer to the charge on the molecule.

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photolysis of frozen aqueous solutions at low temperatures.¹¹ The mechanism was shown to be photolysis of the metastable triplet state of these species. However, for the pyrimidine compounds, e.g., thymine and cytosine, cation radicals are not produced at pH 7. In agreement with these results Shulman and coworkers found through both optical and esr methods that the neutral thymine and cytosine molecules did not show phosphorescence or appreciable population of the triplet state.¹²⁻¹³ By increasing the pH to 12 where thymine loses its N_3 proton, phosphorescence and esr signals due to the triplet were observed by these workers with a decay time of 0.60 sec. From this previous work it is reasonable to expect that under conditions of high pH and low temperature thymine should photoionize from its excited tripled state.

ESR studies of the uv photolysis of thymine have shown that thymine does photoionize at 77 K in alkaline aqueous glasses such as 5 M K_2CO_3 , 8 M NaOD or basic 8 M $NaClO_4$.^{14,15} Perhaps unexpected from the previous work the thymine π -cation is produced by photolysis in an acid glass (5 M D_3PO_4) as well.

In Table I the triplet lifetimes at 77 K reported by Shulman and Rahn for a number of DNA constituents are shown.¹²

Table I. Triplet Lifetimes (τ) at 77 K

Compound	τ (sec) ^a	pH
Thymine	0.6	12
Thymidine	0.6	12
TMP	0.5	12
Cytosine	-	7,12
GMP	1.26	7
AMP	2.4	7

In general it has been found that the DNA bases, nucleosides or nucleotides can be photoionized at 254 nm in aqueous glasses if they have triplet lifetimes on the order of those shown in the table and significant intersystem crossing.

The aqueous glasses that have been employed in these studies are prepared from 8 M NaOD, 5 M D_3PO_4 , 8 M $NaClO_4$, 12 M LiCl, 5 M K_2CO_3 or 50% glycerol in H_2O by cooling these solutions to 77 K.^{14,15} Inorganic glasses are preferred since matrix radicals are less likely to form. Glasses prepared from 8 M $NaClO_4$ have proven most useful in the study of cations due to the fact that the photoejected electron is scavenged by ClO_4^- as in reaction 1

The broad ESR signal from O^- can be easily subtracted from the π -cation signal with computer techniques.

B. The Formation of DNA Base Anion Radicals by Electron Attachment

Esr studies of the reaction of electrons with DNA bases and nucleotides in alkaline and neutral aqueous glasses have been helpful in elucidating the role of the electron in radical production.^{7-9,14,16} In our work electrons are produced by the uv photolysis (254 nm) of $K_4Fe(CN)_6$ ($10^{-2}M$) in a number of aqueous glasses at 77 K.¹⁷ The DNA bases are kept at $10^{-3}M$ or lower to prevent photoionization. The photo-oxidation of ferrocyanide produces ferricyanide. Ferrocyanide is not paramagnetic and ferricyanide does not interfere with the $g=2$ region of magnetic field that is of interest. The glasses we have found most useful in studies of electron attachment reactions are prepared from 8 M NaOH and 12 M LiCl. Matrix radicals, chiefly Cl_2^- , are produced in LiCl by photolysis only if the concentration of $K_4Fe(CN)_6$ is low. Deuterated solvents are employed to reduce depolar broadening and improve resolution.

Immediately after photolysis the sample is photobleached with light from an incandescent lamp to mobilize electrons trapped in the glass. The photoproducts and photobleached electrons either react with the solute or the ferricyanide formed during the photolysis.

This technique has been employed successfully in a number of studies. In the following section we describe perhaps the most significant of these studies, the investigation of electron reactions with dinucleoside phosphates.⁷

C. π -Anions of Dinucleoside Phosphates

An ESR and pulse radiolysis study of the reaction of electrons with dinucleoside phosphates (DNPs) was begun to better understand the role of the electron in DNA radiolysis. Specific goals were 1. to ascertain on which DNA base the electron localized and 2. to determine what reactions occur after formation of the DNP anion.⁷

It is important to the first goal to learn whether DNA bases in DNPs are stacked so as to make electron transfer to the more electron affinic base possible in the rigid glass used in the ESR study or in the aqueous solution used in the pulse radiolysis study. There are a number of investigations

Figure 1. First derivative ESR spectra of the nucleoside anions in 12 M LiCl-D₂O at 110 K: (T) thymidine anion (dC) deoxycytidine anion, (dA) deoxyadenosine anion, (dG) deoxyguanosine anion. The distance in magnetic field between the markers in the spectrum is 13.0 G. The central marker is at $g = 2.0056$.

Figure 2. The ESR spectrum (lower spectrum) of the TpdA anion in 12 M LiCl-D₂O at 110 K. The ESR spectrum (upper spectrum) found for T anion. Computer simulations which summed the dA anion with the T anion gave a best fit for pure T anion.

Figure 3. The ESR spectrum (lower spectrum) found after the reaction of electrons with an equal molar mixture of T and dA in 12 M LiCl at 110 K. Computer simulation (upper spectrum) of the lower curve summing T anion and dA anion in the ratio of areas 6:4.

which have shown that the DNA bases in dinucleotides, or free solution, will tend to stack.¹⁸⁻²⁰ The stacking has been found to be temperature dependent with stacking favored at lower temperatures. Purine-purine stacking is found to be favored somewhat over pyrimidine-purine stacking. In this ESR work the room temperature solutions of DNPs in 12 M LiCl are cooled to 77 K. But the solution remains a liquid upon cooling to approximately 190 K where the solution becomes a glass. Thus, in this system stacking should be greatly favored. The high salt concentration in the solution might be expected to affect the stacking; however, Brahms, Maurizot and Michelson have shown in work with a number of DNPs in 5 M KI that the ionic strength had no important effect on the stacking.²⁰

The ESR spectra of the anion radicals of the four DNA nucleosides in 12 M LiCl-D₂O at 110°K produced by uv photolysis of K₄Fe(CN)₆ are shown in Figure 1. These spectra were used to simulate the spectra of the DNP anions discussed below. The analyses of the T, dC, adenine and guanine anion spectra have been reported elsewhere.^{9,14,21,22}

In Figure 2 we show the result of electron attachment to TpdA. Computer simulations show the best fit to be 100% T anion (shown in Figure 2). Simulations with as little as 10% dA anion were distinguishable. This results suggests that the DNA bases are stacked and the electron is transferred from dA to T. Another possibility is that they are not stacked and the electron reacts much more readily with T than dA in the DNP. This possibility is not in accord with the kinetics of electron reaction with thymine and adenine in aqueous solution.²³ These results show the rates of reaction to be comparable.

Since it could be argued that the rates may differ in a frozen glass of high ionic strength a test of the second explanation was performed. In these experiments equal molar mixtures of T and dA (1×10^{-3} M) were prepared. Electron addition gave the spectrum shown in Figure 3. The computer simulation gave a best fit for 60% T anion and 40% dA anion. This result is quite reasonable in light of the previous kinetic studies. The combined results for TpdA and T + dA strongly suggests that electron transfer to T is occurring with in the DNP.

Electron reactions with TdG and T plus dG mixtures also suggest electron transfer from the purine, dG, to the pyrimidine, T. Electron reactions with TpdC and dApdC were not as conclusive, but show that T and dC share the excess electron equally in TpdC anion whereas the electron resides principally on dA in dApdC anion.

The pulse radiolytic work confirmed that electron transfer occurs from dA to T in TpdA anion.

Our results show that electron transfer occurs in the DNPs from the purine to the pyrimidine base at 77 K. This initial unpaired electron distribution in the DNPs is a consequence of the relative attraction of the DNA bases for an excess electron. Thus the ESR results found for the DNP anions can be used to order the four nucleosides in terms of their electron affinity in an aqueous solution. The results for TpdA and TpdG clearly show that the electron affinity of T is greater than dG or dA. Although the relative ordering of the electron affinity of dC was not unequivocally determined, the results suggest that it is intermediate between T and the purine nucleosides. Thus, the following order of electron affinities for all the DNA nucleosides is indicated:

$$T = dC > dA \approx dG$$

The ordering of the electron affinities of the free DNA bases would be expected to be the same as found in the DNPs.

Our results are in agreement with theoretical calculations of the electron affinity of the DNA bases.^{24,25} These calculations predict the order of electron affinity to be thymine > cytosine >> guanine > adenine. Experiments on the radiation chemistry of DNA or DNA bases also lend some support to our findings.

D. π -Cations in DNA Constituents and DNA

π -cation radicals of DNA bases have been identified in γ -irradiated crystalline thymine²⁶, thymidine²⁷, cytosine²⁸ as well as DNA¹ itself. The π -cation radicals of all the DNA bases save cytosine have been produced by photoionization in aqueous glasses.^{9,11,14,15,21} The purine cations show relatively unresolved spectra⁹ while those of thymine and 5-methyl cytosine show well resolved ESR spectra which have been fully interpreted.¹⁵ Recent work²¹ with π -cations of TMP, and thymidine has resulted in spectra which show a resolved coupling to the ribose group. In Figure 4A the spectrum of the TMP π -cation in 8 M NaClO₄ is shown. The parameters used in the reconstruction given in Figure 4B are $a(\text{CH}_3) = 21.3\text{G}$, $a(\text{ribose } \beta\text{-proton}) = 8.3\text{G}$, $A_{||}(\text{N}_1) = 13.1\text{G}$, $g_{||} = 2.0022$ and $g_{\perp} = 2.0040$. The methyl group and nitrogen splittings are not found to vary significantly with the substituent at N₁. In fact the thymine π -cation itself shows values of these splittings very near those reported above.¹⁵

In our most recent work we have performed a study of various dinucleoside phosphate and DNA π -cations. Photolysis of these molecules in 8 M NaClO₄ at 77 K has resulted in the finding that principally the guanine π -cation is stabilized in DNA or in DNP's containing guanine. In the following we illustrate our findings for DNA. Figure 5A shows the spectrum of DNA in 8 M NaClO₄ immediately after photolysis. The low field signal is due to O⁻ formed by reaction of the photoejected electron with ClO₄⁻ (reaction 1). In Figure 5B we show the spectrum of O⁻ itself. The subtraction of B from A is shown in Figure 5C. Double integration of A and B showed that about 50% of the radical was due to O⁻. This suggests the signal observed in Figure 5C is due only to the DNA π -cation. In Figure 5D we show the guanine π -cation from the photoionization of GpG. The photoionization of dG produced an identical spectrum. The spectrum in Figure 5D is virtually identical to that found for DNA as evidenced by the subtraction of 5D from 5C shown in Figure 5E. The above evidence combined with the fact that the spectra found for the π -cations of T, dC (from single crystal work) and dA are distinguishable from that of dG leads us to the conclusion that the π -cation in DNA is localized largely on guanine.

We believe there are two possible interpretations of the results found for DNA. First the observation of the guanine π -cation may simply be due to its selective photoionization. In agreement with interpretation it is found that guanine is somewhat more easily photoionized than adenine whereas the pyrimidines can not be photoionized in neutral glasses. However, under basic conditions where thymine can be photoionized still only the π -cation of guanine is observed. Interestingly the signal due to the π -cation is much more intense in basic solutions. The second possibility is that photoionization from guanine, adenine and thymine is followed by hole transfer to the DNA base with the lowest ionization potential (guanine). Since hole transfer through the stacked DNA bases is likely to occur and since on the average a guanine base should be only a few DNA bases from the original hole site, this mechanism has the most appeal.

The report of Shulman and Rahn that the phosphoresence from thymine containing dinucleotides and DNA is from the thymine base alone is pertinent to this point.¹² They suggest that the triplet excitation is transferred through the DNA chain to the base with the lowest triplet energy (thymine). The build up of the thymine triplet state is of course a prerequisite to the photoionization of thymine. From the above it seems a major fraction of the cation radicals should be produced on thymine. However this was not found to be the case.

Figure 4. (A) The first derivative ESR spectrum of the TMP (thymidine-5'-monophosphate) π -cation radical in a basic 8M NaClO_4 - D_2O glass at 115 K, produced by photoionization. The O^- spectrum accounts for the broad low field and the central underlying components. (B) Computer simulation of the π -cation spectrum in A employing parameters in text.

Figure 5. (A) ESR spectrum of salmon sperm DNA after photoionization in 8 M NaClO_4 (B) The spectrum of O^- in 8 M NaClO_4 (C) $A - 0.5 B$ (D) The spectrum of the π -cation of GpG in 8 M NaClO_4 (E) $C - D$

This combined with the fact that only the guanine π -cation is thought to be present in γ -irradiated DNA, points to hole transfer within the DNA strand.

E. Reactions of the Ion Radicals in DNA and DNA Constituents

The results presented here and those of previous workers suggest that the initial localization of charge on the DNA strand after an ionization event is likely to place the excess electron on thymine and the hole on guanine. The reactions which take place subsequently will of course depend on environment. However results with DNA and DNA constituents have shown the following reactions occur in aqueous matrices at low temperature.

1. Protonation of the thymidine anion at the 6-carbon position to form TH $\cdot$.6,10,16
2. Protonation of the dAMP and dGMP anions at the 8-carbon position to form AH $\cdot$ and GH $\cdot$.⁵
3. Protonation of solely thymine in dinucleoside phosphate anions containing thymine and the other DNA bases to form TH $\cdot$.⁷
4. Deprotonation of the thymidine cation methyl group to form T(-H) $\cdot$.⁶
5. Hydroxyl ion addition to the thymidine cation at the 6-carbon position to form TOH $\cdot$.4,8
6. Hydroxyl ion addition to the dAMP and dGMP cations at the 8-carbon position to form AOH $\cdot$ and GOH $\cdot$.⁵

In γ -irradiated DNA the TH $\cdot$ radical has been observed by a number of workers and has been shown to have the thymine anion as its precursor.² There has been one report of the T(-H) radical in DNA,²⁹ however its presence is not as well established as TH $\cdot$. Although the fate of the cation in DNA is unclear, the deprotonation and hydroxylation mechanisms noted above seem most likely to explain the subsequent reactions of the hole in DNA at this time.

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