CHAPTER - 2

REVIEW
REVIEW

REVIEW OF THE RADICAL INDUCED DAMAGE TO THE CONSTITUENTS OF NUCLEIC ACIDS.

It is widely believed that oxidative damage to DNA plays a crucial role in carcinogenesis. Damage to DNA in cells has been found to be the major cause for mutation, cancer and cell deactivation under the influence of chemicals, UV light and high energy irradiation. It is known that ionising radiation [1,2] causes a number of deleterious effects ranging from reproductive cell death to mutagenesis and transformation. These effects are mainly due to lesions induced in cellular DNA, which is believed to be the prime targets for the action of ionising radiation, both via direct ionisation of the nucleotide bases [3,4] and indirectly from secondary damage via reaction of "OH (from water, 5-6). Over the period, much of the progress in this field has developed in parallel in two distinct areas that have remained essentially independent of each other. One is the realm of (aqueous) solution chemistry where product analysis and pulse radiolysis [7-8] have predominantly been applied and the other field is that of solid state (including frozen solutions) ESR [9-10]. It
is well understood that it is the radical intermediates which play a key role in the mechanism of the induced damage either chemically or through ionising radiation. Ever since the early days of radiation chemistry and biology it has been realised that the purine and pyrimidine bases are the most sensitive to radiation induced modification or destruction of the components of DNA, itself the most critical of the cellular targets [11-13]. Attempts to understand the radical chemistry leading from the primary ionisation event to the final non-radical products have involved the use of three general "tools":

a. Product analysis studies on, mainly, the building blocks of DNA and model compounds for them [14-17].
b. Electron spin resonance in matrices, single crystals and liquid solutions (spin trapping and flow techniques) [18,21].
c. Time resolved methods (pulse radiolysis) with predominantly optical and conductance detection [22-25] again mainly on the constituents of DNA and their model compounds. The study related to Radiation induced damage to DNA by using various "tools" can be broadly divided into two parts. One comprising of the solid state and the other of the solution state.

2.1 SOLID STATE STUDIES:

Most solid state studies have centered upon Electron Spin
Resonance (ESR) and Electron Nuclear Double resonance (ENDOR) techniques. The applicability of information derived from the solid state studies to the in-vivo system is often questioned. But, first the state of DNA in vivo lies somewhere between fluid and solid. To understand radiation effects in vivo, it is necessary to interpolate between the results from both liquid and solid samples. Second, solid state studies are informative about processes that would occur very rapidly in liquids, processes involving ionic intermediates, unimolecular events, and small highly reactive fragments.

PYRIMIDINE BASES

A. Electron abstraction: The loss of an electron by a pyrimidine base leaves a vacancy in the HOMO of the system. The resulting radical has been described most accurately as a π cation by Sevilla and co-workers [26] because that description leaves undesignated the net charge of the radical. The unpaired electron remaining in the HOMO has its highest density at C5. Marking off alternate atoms from C5, other sites of unpaired electron population can be anticipated: N1, N3 and the exocyclic groups at C2 and C4. Deprotonation is possible at position α or β to the sites of highest unpaired electron population in pyrimidine cations, C5 is largest and N1 is next largest [27]. The
most probable routes to deprotonation are shown in the following scheme, 1:

It has been pointed out that the unsubstituted Uracil or Cytosine will undergo deprotonation at N1, and if there is a substituent at N1 deprotonation will occur from the substituent. In crystalline Thymine matrices, the cation deprotonates at N1 or the methyl group. Sevilla et al. studied N1 substituted Thymine cation radicals in glasses, they obtained convincing evidence that the N1 and not N3 is the major source of nitrogen hfs. Although for all the pyrimidines studied the site of deprotonation is the N1, when there is a substitution at N1, the site of deprotonation is still keyed to N1. Deprotonation is, however, β to N1 and not α.

Anion addition: Anion addition is an alternative mechanism by which a pristine cation can return to the parent charge state. Anion addition replaces the lost electron with two electrons, resulting in structures analogous to electron addition radicals. For example, OH⁻-addition to C6 of the pyrimidine cation gives a radical analogous to radicals formed by protonation of C6 of anion.

The addition of OH⁻ to Thymine cations has been demonstrated by Sevilla and Engelhardt. They suggest that the change in the H6 hfs, αβ hfs, comes from a change in the tortional angle. Their idea is that, at increased
SCHEME - 1
temperature, the OH group moves from a nearly axial to a more equatorial position. H6 must then become more axial and \( \theta \) smaller.

B. Electron Addition:

Since the excess electron is in a \( \pi \) orbital, these ions are called \( \pi \) anions, regardless of the protonation state. The unpaired electron resides primarily at C6, C4 and C2. The position of highest spin density C6, is emphasised in drawing the valence bond structures A and B. Excess negative charge is expected at N3 and O2 for Cytosine and O4 and O2 for uracil. In both Cytosine and Uracil radicals the other mesomeric structures carry the formal negative charge to C5.

Protonation pathways are shown in the Schemes 2,3. It has been demonstrated that protons are attracted to positions that have undergone the greatest increase in negative charge. Predicting the position requires the consideration of the source of proton. The proton could be donated by a hydrogen bond donor group, or it could be a "free" proton generated by decomposition of an electron loss centre.

Bernhard points that hydrogen bonded protons are most likely
SCHEME - 2
SCHEME - 3
to transfer to N3 of Cytosine anions and O4 of Uracil anions. In both cases, it is also possible to transfer a proton to O2; but O2 in crystals at least, does not usually participate in strong hydrogen bonds, thereby reducing the opportunity for this mechanism to operate. For the Thymine anion, it is unclear whether or not a proton transfers across an H bond to add at O4 or O2. It is known that the radical can acquire a matrix position at C6. This can definitely occur after protonation at O4 and probably also before protonation at O4.

He further points that "Free" protons will add either to O2 or to the C6 = C5 aromatic bond of the pristine anion. A probable site of addition is O2 if its lone pair orbital is not occupied in a strong H bond. For the Thymine it is the C6. And for Uracil and Cytosine, this position is unknown but it could be C5. If the pristine anion is already protonated at N3 in Cytosine, or at O4 in either Uracil or Thymine, addition of the free proton to the C6 = C5 bond will result in a net gain of H at C6. The resulting radical will have a positive charge.

Currently it is not clear whether any of the reported anions are in fact pristine anions. In every case protonation at the heteroatom allows alternatives. There is however, good indirect evidence which indicates that heteroatoms are readily protonated in the temperature region
77-300 K. Sevilla et al have provided univocal evidence for C6 as the main site of unpaired electron density.

C. Excitation and Hydrogen Atom Reactions:
Superexcitation in unsubstituted Uracil or Cytosine appears to lead to dissociation of either the C6-H6 or the C5-H5 bond. However, if a methyl group is attached to N1, an H atom will be lost from the methyl group. The H atom thus produced adds preferentially (perhaps exclusively) to C5. Apparently, addition at C5 occurs whether or not a proton is at N3 in Cytosine and O4 in Uracil. The superexcited state of Thymine, substituted at N1 or not, will lose an H atom from the C5 methyl group. The resulting H atom will add to C6, specifically, whether O4 is protonated or not. The following Schemes 4,5 show these pathways for Cytosine and Thymine respectively.

PURINES

A. Electron Abstraction:
Electron loss from Guanine or Adenine should lead to deprotonation or anion addition reactions; however, predicting the sites of deprotonation is difficult because the unpaired electron distribution is diffuse. Examination of the resonance possibilities for each of these forms shows that the unpaired electron is located not only on C2 and C4 but also C5, N1 and N3. ESR-ENDOR analysis shows that
SCHEME 4

SCHEME 5
imidazole portion of the purine ring, the result would be to equalise the unpaired spin in the -C = C- double bond in contrast to pyrimidines.

Deprotonation of the cation in unsubstituted adenine occurs at N9 or N6' and in unsubstituted Guanine at N9, N1, or N2'. Substitution at N9, leads to a proton loss from that substituent at a position to unpaired electron density at N9 (schemes 6, 7). Return to the parent charge state can also occur by anion addition to C8.

B. Electron addition:
The model for electron addition to Adenine, have shown that protonation via hydrogen bonds is most probable at N1 and N7, with N1 favoured over N7. Protonation via "free" protons occurs at non-hydrogen bonding orbitals, usually N7 or N3 and at the carbons C8 or C2, depending on the prior protonation state at N7, N3 and N1. Protons add preferably to C8. This event is more probable, or at least the resulting radical is more stable, if a proton is on N7 or N3. A proton on N1 stabilises the C2 addition radical and possibly promotes protonation at C2 of the pi anion. Conversion between the C2 and C8 H adducts depends on the protonation state of the radical as shown below in the scheme 8.

C. Excitation and Hydrogen Atom Reactions:
SCHEME - 6

SCHEME - 7
The site of H atom attack is C8 and/or C2 in Adenine and C8 in Guanine. Accordingly a substituent on N9 is a good source of H atoms. In the absence of an N9 substituent, the C2-H bond of Adenine is a site of homolytic dissociation. For Adenine, the expected reactions are shown in Scheme 9.

DNA MACROMOLECULE

DNA exposed to the direct effects of ionising radiation undergoes chemical changes that originate primarily from sites that have either lost or gained an electron. Determining the chemical nature and the distribution of the damaged sites is a central problem in the field of radiation biology.

The most widely accepted model, at the present, is that at temperatures of 77K and below, electrons are trapped predominantly (or exclusively) at Thymine and holes are trapped at Guanine [29]. But difficulties with the T\(^-\)/G\(^+\) model of direct damage in DNA have been apparent for some time. Graslund et al in their work on fiber DNA were unable to eliminate C as a site that traps electrons, in addition to T [30]. Sevilla et al, studying dinucleoside phosphate, concluded that the pyrimidines are more electron affinic than purines but could not exclude the possibility that cytosine's affinity was comparable to Thymine. In summary, there is evidence that electron attachment to the pyrimidine
bases is more probable than to the purine bases, but the relative distribution among the four bases is unknown. It has been shown through ESR that Cytosine is also a dominant site of Electron trapping [31].

In summary it can be said that ESR studies of irradiated solid DNA and its constituents at 77 K have suggested the formation of one electron deficient (a radical cation) and one electron rich (a radical anion) center on the bases but not on the phosphate [32, 33]. There is a general agreement that the positive charge migrates to and is localised in Guanine. Earlier it has been suggested Thymine as a final location of negative charge [32]. However recent studies have questioned this assignment and proposed Cytosine instead [34, 35].

2.2 SOLUTION STATE STUDIES:

Since ionising radiation absorption is not specific to special residue(s) of the molecule, chemical transformation (radical cation formation and solvated electron) can occur in principle, on any constituent of DNA (nucleobases, sugars or phosphates) [36]. DNA lesions are partly generated also by indirect effect where the radiation is absorbed by the solvent (water) in close proximity to form the oxidising OH radical and the reducing hydrated electron (e⁻aq.) and H' [37].
A. Reaction with OH':

It is generally accepted that 'OH radical is the most reactive of them, and has been shown to react [38-41] with pyrimidine such as uracil, thymine etc.. The reactions are generally accepted to involve additions to the heterocyclic systems i.e., the major site of initial damage, induced by both 'OH and direct ionisation is the nucleotide base, which then results in the transfer of damage from the base to the sugar phosphate [42-48]. The mechanism of these transfer processes are not clear, though under certain circumstances, base radical cations may be involved [49-51]. It has been demonstrated that the hydroxyl radical combine with the 5,6 double bond of the pyrimidine base moiety to form C5-yl and C6-yl radicals and abstract hydrogen atom from the sugar moiety to form carbon centered radicals [52]. The 'OH induced C5-yl and C6-yl radicals are regarded as precursors of base damage [53]. However, the mechanism of transfer of the site of radical attack on the pyrimidine to the sugar ring, as a prelude to fragmentation via phosphate loss is not clear, though under certain circumstances radical cation may be involved [54] followed by rapid transfer of the radical centre to C2' in the ribose ring. The reaction of 'OH with pyrimidine bases may be summed up as shown in scheme 10;
SCHEME - 12
and with the pyrimidine nucleosides as, scheme 11;
while addition of 'OH with purine bases may be as, scheme 12;
whereas with purine nucleosides, the initial site of attack
may be transferred from the base to the sugar in a similar
way depicted for pyrimidine bases.
B. Reactivity with e\textsuperscript{-aq}:
The purines have a very high intrinsic reactivity with e\textsuperscript{-aq}
[40]. This property is endowed with the electron deficient
pyrimidine. All purines react as neutral bases with e\textsuperscript{-aq}
with second order rate constants, essentially independent of
their individual structure. In the past there have been only
few attempts aimed at elucidating in detail the nature and
further reactions of purine electron adducts. Notable among
them are the investigations of Moorthy and Hayon [55],
Sevilla et al. [56] and Hissung et al. [57]. They found that
the electron adducts change with pH in a way that was
interpreted in terms of protonation equilibrium of the e\textsuperscript{-aq}
adducts. Hissung et al. were able to demonstrate by
conductance and optical measurements that the electron
adduct is mono protonated (i.e it is a neutral radical).
This conclusion has been confirmed by Visscher et al. [58,
59]. From ESR spectroscopy of purine radicals, it is known
that the electron adduct to the adenine moiety (the "
pristine anion") gets protonated (even at 4K) [60-62]
on a nitrogen and upon warming, the reaction is followed
(via paths not very well understood) by a rearrangement that results in protonations at carbon 2 or 8 of the purine system.

For the pyrimidine bases, the unpaired electron resides primarily at C6, C4 and C2. Excess negative charge is expected at N3 and O2 for cytosine and at O4 and O2 for uracil [60],

For protonation pathways, it may be mentioned that the protons are attracted to positions that have undergone the greatest increase in negative charge. If a proton is available at time of addition and protonation will be fast if the required activation energy can be obtained. Since, the excess electron is in a pi orbital, these ions are called pi anions, regardless of protonation states.

C. Electron abstractions:
Reactions of SO4^−, Br2^−(the secondary radicals) are reported in literature [53-61, 63-71]. Fujita et al have also used other secondary oxidising radicals in addition to these, they are Cl2^−, (CNS)2^− etc. [72]. With the
pyrimidine system the electron abstraction can be shown to occur as,

\[
\begin{array}{c}
\text{H-N} \\
\text{O} \\
\text{O} \\
\text{H-N} \\
\text{SO}_4^+ \\
\end{array}
\rightarrow
\begin{array}{c}
\text{H-N} \\
\text{N} \\
\text{O} \\
\text{H} \\
\end{array}
\rightarrow \text{UNDERGOES DEPROTONATION}
\]

Although sites of deprotonation are many e.g., when the Thymine moiety is ionised deprotonation is possible from N1 [ 73 ] N3 and from the methyl group at C5 [ 40 ] and also hydration at C5-C6 [ 46,74,75 ]. Deprotonation can thus in short occur at positions \( \alpha \) or \( \beta \) to the sites of highest unpaired electron population. For the purine bases, electron loss leads to deprotonation or anion addition reactions, however, predicting the sites of deprotonation [ 40 ] is difficult because the unpaired electron distribution is diffuse. The purines have odd numbered rings, so the simple picture for pyrimidines, that of unpaired electron density at alternate ring positions, no longer applies. However, examination of the electron population should be located at C2, C4, C5, N1 and N3. Steenken et al. have shown the reaction of \( \text{SO}_4^2- \) with adenine to proceed as:

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{H} \\
\text{N} \\
\text{N} \\
\text{C} \\
\text{R} \\
\end{array}
\rightarrow
\begin{array}{c}
\text{H}_2\text{N} \\
\text{N} \\
\text{N} \\
\text{C} \\
\text{R} \\
\end{array}
\rightarrow
\begin{array}{c}
\text{H}^+ \\
\text{N} \\
\text{N} \\
\text{C} \\
\text{R} \\
\end{array}
\]

60
In addition N9 has also been shown to be a site of deprotonation. If there is a substituent at N9, a proton can be lost from that substituent at a position $\beta$ to unpaired electron density at N9.

These damages produced in the heterocyclic bases are then transferred to the sugar leading to strand breakage - one of the major lesions to cause damage to DNA. Care has been taken to give proper credit for the works of other authors in the literature. The author would like to apologise for any omission which may have occurred by oversight or error in judgement.
REFERENCES


(13) Von Sonntag, C.; The Chemical basis of Radiation Biology; Taylor and Francis, London (1987)


(18) Box, H.C.; Radiation Effects, ESR and ENDOR Analysis; Academic: New York (1977)


(27) Kar, L.; and Bernhard, W.A.; Advances in Radiation Biology, Vol. 9, p. 212.


(37) No 13 and the references therein.

(38) No 11 and the references therein.


(52) Kuwabara, Minikora Radiation Phys. Chem. Vol 37, 5-6, Pg 691-704 (1991)

(53) No. 13

(54) No. 39

(56) Sevilla, M.D.; Failor, R.; Clark, C.; Hairoyd, R.A.; Pattei, M.;

(57) Hissung, A.; Von Sonntag, C.; Veltwisch, D.; Amus, K.D.;

(58) Visscher, K.J.; de Haas, M.P.; Loman, H.; Vijnovir, B.;


    Magnetic Resonance of the Solid State. J.A. Weil,

(63) Javanovic, S.V.; Simic, M.C.; J. Phys. Chem. (1986) 90, 974-
    978.

(64) Myers, L.S. Jr; Hollis, M.L.; Theard, L.M.; Peterson, F.C.;


    4138-4144.

    587.

(69) Buxton, G.V.; Greenstock, C.L.; Helman, W.P.; Ross, A.B.;

66


OBJECTIVE
OBJECTIVE

Damage to DNA has been a major area of research. This damage is primarily responsible for cancer, aging, cell deactivation etc. Although, a lot of work on damage related studies have been done, the real cause of cell deactivation is still not precisely known and debate is going on. These studies have been mainly confined to DNA and its constituents, in the solid state or solution state, mainly the aqueous state. These studies have been undertaken at:

(i) at 77K or even at 4K,
(ii) Using a powerful source of irradiation e.g., γ-rays, pulse radiolysis, photoexcitation etc.,
(iii) Reaction with strong oxidising agent e.g., $\text{SO}_4^{2-}$, $\text{OH}^-$ etc.,
(iv) in aqueous phase.

In spite of so much of work, the mother nature has illuded the scientific community and kept up to herself the greatest secret of all - the cause of cancer. The present author, therefore, felt strongly motivated to undertake the study with a different approach with a hope that our findings, no matter how trivial they may be, might help towards a wider understanding of the problem. We, therefore, confined our work:

(i) at ambient temperature, because the human body is at that temp. Any damage at this temp. will be relevant to the
system in vivo.

(ii) Not employing any powerful irradiating source of radical generation. Because the percentage of cancer deaths due to radiation exposure is very low.

(iii) By creating such conditions where initially only one electron is transferred and the chain of reactions begins.

(iv) By employing spin trapping technique to trap the short lived intermediates.

(v) Non-aqueous phase. It is known that some very fast two electron reactions in aqueous phase can proceed in two steps involving one electron reaction in non-aqueous solvents. Moreover, the non-aqueous phase provides an ideal environment which mimics the interior environment of double helix of DNA.

(vi) If an electron transfer occurs under these conditions, does it follow an "Inner - sphere or Outer - sphere" mechanism.

The present project has been executed in two parts. First part deals with the electronic spectroscopic studies of the nucleic acid bases in non-aqueous solvents. In order to understand the role of any molecule in single electron transfer processes in a given media, it is necessary to understand its optical properties in those solvents. Literature survey revealed that so far the uv spectra of these base molecules in non-aqueous solvents has not been
studied so far. Therefore, it became imperative to study the uv spectra and its associated properties in solvents of different polarity. It is also accepted that for a molecule to participate in electron transfer processes, the primary requirement is that a molecule should form a charge transfer complex with that molecule to whom electron is to be transferred. UV spectroscopy is a simple technique through which these informations can be obtained satisfactorily.

The second major part of the project is the study of single electron transfer reactions by ESR (employing spin trapping technique).