THE ONE-ELECTRON ADDBUTS OF PYRIMIDINE BASES IN LOW-TEMPERATURE GLASSES AND THEIR SIGNIFICANCE TO γ-IRRADIATED FROZEN AQUEOUS DNA: AN EPR STUDY

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By

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STATEMENT

The experimental work described in this thesis has been carried out by the author in the Department of Chemistry at the University of Leicester between October 1986 and January 1990, unless otherwise acknowledged in the text. The work has not been submitted, and is not currently being submitted, for another degree at this or any other University.

Signed: ........  Date: ....3.13.93....
Dedicated to Mum, Dad and Karen for their continual support and encouragement
ACKNOWLEDGEMENTS

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IAN D. PODMORE

ABSTRACT

By the use of model compounds such as pyrimidine bases, nucleosides and nucleotides in low-temperature aqueous glasses, the primary reduction product of $\gamma$-irradiated frozen aqueous DNA has been examined using EPR spectroscopy. Ionisation of frozen aqueous solutions of lithium chloride, sodium chloride, methanol, ethylene glycol and sodium hydroxide generates positive 'holes' and mobile electrons. In the absence of suitable solutes, such as DNA bases, both species are trapped within the glass. In the presence of pyrimidine derivatives, however, the mobile electrons react to form $\pi^\ast$-anions.

For thymine and uracil derivatives the $\pi^\ast$-anions, as monitored by EPR spectroscopy, give rise to anisotropic doublets ($A_{\text{iso}} \sim 12-14\text{G}$) in both $H_2O$ and $D_2O$ matrices (chapters 2 and 4 respectively). For cytosine derivatives, however, although doublets are observed in $D_2O$ ($A_{\text{iso}} \sim 13-14\text{G}$), triplets are detected in $H_2O$ systems corresponding to an extra 12G splitting (chapter 3). This splitting is assigned to a proton attached to the exocyclic amino nitrogen atom (N4) and is taken as clear proof of heteroatom protonation of the radical anion of cytosine. No direct EPR evidence for the protonation states at 77K of either the thymine or uracil $\pi^\ast$-anions is obtained in any of the glasses. On warming these systems, however, protonation of the $\pi^\ast$-anions of both thymine and uracil at a carbon atom position (C6) is observed giving rise to C6 H-adduct radicals. No such species is detected for cytosine on annealing.

Exposure of frozen aqueous DNA to $^{60}\text{Co} \gamma$-rays at 77K gives electron-loss and -gain centres localised on the bases. The electron-gain centres are believed to be a mixture of pyrimidine $\pi^\ast$-anions (Py$^\ast$, i.e. C$^\ast$ + T$^\ast$). This assumption is based on the fact that, on annealing, the Py$^\ast$ doublet is only partially converted into the 5,6-dihydrothymine-5-yl radical TH, the resulting radical having a completely characteristic octet EPR spectrum. The results suggest that ca. 36% of the doublet is due to T$^\ast$ centres, the remainder (64%) being assigned to C$^\ast$ centres. It is argued that ejected electrons move through stacked bases, becoming trapped at cytosine or thymine depending upon the relative rates at which C$^\ast$ and T$^\ast$ are protonated to give C$^\ast$(H$^+$), protonated at N3 (not N4), and T$^\ast$(H$^+$), probably protonated on a carbonyl oxygen. For this to be correct then interconversion between C$^\ast$ and T$^\ast$ on annealing is unlikely.
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DNA Structure and Ionising Radiation
1A.1 INTRODUCTION

Ionising radiation has become an important part of human life during the 20th century. Radiation and radioactive materials are now used extensively in medicine, industry and power generation. One of the many problems associated with their use is the possible threat of nuclear accidents. Indeed, this threat has already become a reality. The accident at the Chernobyl Nuclear Power Station, in what was the Soviet Union, on the 26th April 1986, is the worst of its kind to date. Of the 135,000 people evacuated from within a 30km radius of the accident site, some 24,000 are believed to have received radiation doses that may ultimately produce radiogenic disease (Pravda, 1986; U.S.S.R. Committee on Atomic Energy, 1986).

Radiation damage has profound biological effects on many types of cells and organs. The identification of the principle target molecule whose damage may be responsible for radiation induced cell injury has been the subject of many investigations and, in mammalian cells, is believed to be that of deoxyribonucleic acid, DNA (Kaplan and Moses, 1964; Hutchinson, 1966; Alper, 1979; Biaglow, 1981). DNA is the nucleic acid that contains genetic information for all cellular functions encoded within a nucleotide sequence. About two metres of DNA is contained in a nucleus 0.5μm in diameter for a human cell and all this information must be replicated exactly during cell division, as well as be available for transcription and translation. These functions initially depend upon the structure of DNA. Thus, radiation induced alterations to DNA structure, such as single- (SSB) and double-strand breaks (DSB) can lead to mutagenesis or even cell death (Greenstock 1981).

1A.2 THE STRUCTURE OF DNA

DNA is a high molecular weight polymer consisting of two strands and composed of nucleotide building blocks (molecular weight ~ 320). RNA, the second
member of the nucleic acid group, is also a high molecular weight polymer composed of nucleotide subunits, but differs in that it usually consists of one strand. In its messenger form RNA is responsible for translation of genetic information from the cell nucleus to the ribosomes (the site of protein synthesis). Unlike DNA it cannot be found within genes of prokaryotic and eukaryotic organisms but does exist in certain viruses.

A nucleotide consists of a phosphoryl group, a pentose sugar and a heterocyclic nitrogenous base. There are four such bases in DNA; the purines adenine (A) and guanine (G), and the pyrimidines cytosine (C) and thymine (T). Similarly, RNA has three of the bases the same, but the fourth, thymine, is replaced by uracil (U), figure 1A.1. The sugar component in DNA is a cyclic pentose derivative, 2'-deoxy-β-D-ribose. Its parent compound, β-D-ribose, containing no 2'-OH group, is found in RNA, figure 1A.2. Although a small difference between the two nucleic acids, the presence of this bulky hydroxyl group can limit the range of possible secondary structures in RNA and make it more susceptible to chemical and enzymatic attack. Linkage of a base to either a ribose or deoxyribose sugar gives rise to a nucleoside. This glycosyl linkage occurs between the C1' site of the sugar and N1 (for the pyrimidines) or N9 (for the purines). Phosphorylation of a nucleoside at one of the sugar hydroxyl positions (3' and 5' for deoxyribose; 2', 3' and 5' for ribose) yields the nucleotide. Nucleosides and nucleotides are useful DNA model components and are used extensively throughout this study. The nucleotides can be linked by bonding the 3'-hydroxyl position of one sugar to the 5'-phosphate group of the next. Thus, these phospho-diester linkages yield polymeric chains or strands, figure 1A.3.

Watson and Crick (1953a, 1953b) first proposed that DNA consisted of two strands with both of the polynucleotide chains wound around the same axis forming a right-handed double helix. The polynucleotide chains run in opposite polarity (i.e. one is 3'→5' and the other 5'→3') and are held together by hydrogen bonds between the bases. These are formed between a purine and a pyrimidine, with allowed pairings of guanine and cytosine (G-C) and adenine and thymine (A-T), figure 1A.4. Thus, the ratios of G to C and A to T do not vary with the source of DNA. However, it has been shown that the ratio of (G+C) to (A+T) does vary with source (Chargaff, 1963). The most significant consequence of base-pairing in DNA is that the nucleotide sequence of one strand automatically determines that of the opposite strand.

The same base-pairing arrangement is found in all naturally occurring DNA double-helix structures. However, the flexibility in the furanose ring of the sugar (X-ray crystallographic studies by Arnott et al. (1969) and Voet and Rich (1970), and
Figure 1A.1: The structures of the common purine and pyrimidine bases found in RNA and DNA.
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Figure 1A.4. Watson and Crick base-pairing geometry.
NMR solution studies by Jardetsky (1961), show the sugars to be puckered and the degrees of freedom generated by several rotatable single bonds per residue, lead to considerable variation in the conformation adopted by the double-helix structures.

There are two different forms of sugar pucker resulting in small differences in stability. One has four atoms in a plane with a fifth displaced above (or below) the plane and is termed the 'envelope' conformation. The other has three atoms in a plane with two adjacent atoms displaced either side of the plane and is termed the 'twist' conformation. The particular conformation of the sugar ring can be defined in terms of the displacement of the C2' and/or C3' atom(s) from above or below the plane, figure 1A.5. If the major displacement of an atom is towards the same side as the C5' atom the conformation is designated 'endo'. Displacement towards the opposite side is designated 'exo'. Figure 1A.5 shows four of the possible sugar puckers.

The Watson and Crick model has remained close to the DNA structure believed to be prominent in solution and therefore the most important for biological systems. It is found to correspond closely to the structure known as 'B-form'. In this double-helix structure the planes of the base-pairs are nearly perpendicular to the helix axis, and the distance between adjacent pairs along the helix axis is 3.4Å, bringing them into close contact. The structure repeats itself after 10 residues, or once every 34Å along the helix axis. The furanose rings are in the C-2' endo conformation.

When some of the water is removed from the B-form, the double-helices push closer together and the structure changes to the 'A-form' in which the base pairs are tilted ~20° with respect to the helix axis. In the A-form the furanose rings have changed their pucker to the C-3' endo conformation. Due to the bulky 2'-OH group the furanose rings in RNA have a stronger preference for the C-3' endo conformation, with the result that RNA double-helices adopt a structure similar to the DNA A-form even at high degrees of hydration.

Several factors account for the stability of the double-helix structure. The phosphoryl groups are all positioned on the outer surface where repulsive electrostatic interactions generated by their negative charges are often partly neutralised by interaction with positively charged ions (e.g. Na+, the positively charged side of chromosomal proteins, or polyammonium cations such as protonated spermidine). The helical core, composed of the base-pairs, is held together by the hydrogen bonding and also by favourable stacking interactions between the planes of adjacent base pairs. Free bases in aqueous solution illustrate this preference for stacking which arises from interaction between π-electrons of the bases. It should be noted that the DNA double
Figure 1A.5. The four possible sugar ring puckers.
helix is not a totally fixed or rigid molecule. It can undergo a continuous internal deformation, with small segments 'swinging' apart. This phenomenon is known as 'breathing' (McConnell and von Hippel, 1970).

In duplex DNA the helical twist causes there to be grooves in the surface of the DNA helix. Alternating wide ('major') and narrow ('minor') grooves can vary in depth and width according to DNA conformation. Different sections of the purine and pyrimidine bases are exposed in these two grooves. Interactions of proteins with DNA bases occurs in the major grooves due to the greater accessibility.

DNA structure in its functional state is modified through protein binding. This is especially so for eukaryotic DNA where, as already mentioned, an equal weight of protein serves to contract about two metres of nucleic acid into a cell nucleus of approximately 0.5µm in diameter. The resulting DNA-protein complex, which allows access by enzymes involved in all aspects of nucleic acid metabolism, is called chromatin. These chromatin molecules, containing DNA between 10^7-10^8 base pairs in length, are further organised within the nucleus into distinct units called chromosomes. These higher ordered structures of DNA are described in detail elsewhere (Stryer, 1988; Alberts et al., 1989) and will not be discussed further herein.

1A.3 IONISING RADIATION

During the interaction of high energy photons (X- and γ-rays) with matter, most of the energy is absorbed and results in the ejection of electrons from the atoms of the material. This process is almost entirely dependent upon atomic composition and not on molecular structure. In contrast, the absorption of energy from light waves (infra-red, ultra-violet, visible, soft X-rays) generally depends upon the molecular structure of the material and only indirectly on the atomic composition. This is a fundamental difference between ionising and non-ionising radiation.

The consequence of ionising radiation is the formation of free radical ions, such as H_2O^+ formed as a result of the ejection of electrons from the H_2O molecule, scheme 1A.1. H_2O^+ is very reactive and can form neutral free radicals - uncharged molecules with an unpaired electron in an outer orbital, scheme 1A.1. Both free radicals and free
racial ions disrupt normal molecular structures. Therefore, the important result of ionising radiation is the formation of such free radical species.

**SCHEME 1A.1**

\[
\begin{align*}
    \text{H}_2\text{O} & \xrightarrow{\gamma} \text{H}_2\text{O}^+ + e^- \\
    \text{H}_2\text{O}^+ + \text{H}_2\text{O} & \rightarrow \text{H}_3\text{O}^+ + \cdot\text{OH}
\end{align*}
\]

Ionising radiation can consist of either 'directly' or 'indirectly' ionising particles. 'Directly' ionising particles (e.g. electrons, protons and \(\alpha\)-particles) are charged and have sufficient kinetic energy to displace electrons from atoms by collision. 'Indirectly' ionising particles (e.g. photons or neutrons) are uncharged and able to liberate or generate directly ionising particles. Gamma-rays consist of high energy photons resulting from the decay of radioactive nuclei. In the present study \(\gamma\)-rays are the main source of ionising radiation and these are produced by the decay of radioactive \(^{60}\text{Co}\).

Both \(\gamma\)-rays and X-rays can interact with matter in three different ways, and the relative importance of the three processes depends upon the energy of the photons. An example is that of X-ray energies of less than 0.5MeV which interact predominantly by the 'photoelectric' effect. This effect is one in which the photon is completely absorbed by the target atom. The photon's energy is transferred to an inner orbital electron (a 'photoelectron') which is ejected, and replaced by an outer orbital electron, resulting in the production of 'characteristic' energy, figure 1A.6. In order to conserve the momentum carried by the photon the nucleus has to act as a third body. The kinetic energy transferred to the nucleus is only small compared with that given to the electron. The kinetic energy of this ejected electron is equal to the difference between the photon energy and the binding energy of the electron such that.

\[
E_{(e^{'})} = h\nu - E_{(B)}
\]
where $E(e^-)$ is the acquired kinetic energy of the electron, $h\nu$ is the energy of the photon and $E(B)$ is the binding energy of the electron in the atom.

At energies between 0.5 - 5 MeV 'Compton scattering' is the predominant process, figure 1A.6. In this case, a photon collides with an outer orbital electron such that it releases some of its energy to that electron. This 'recoil' electron is ejected. As a result of the interaction, the incident photon disappears and a secondary photon is created with reduced energy,

$$E(e^-) = h\nu - E(B),$$

where $h\nu'$ is the energy of the secondary photon. Depending upon its energy, this scattered photon may then interact with additional target atoms by further Compton scattering or by the photoelectric effect. This process does not require any interaction with the nucleus.

At still higher energies 'pair production' can occur, figure 1A.6. This involves the interaction of photons with the nucleus of the atoms. Such an interaction results in the complete disappearance of the incident photon and the appearance of a positron - electron pair - an example of a physical process in which energy is converted to mass. The rest mass energy of an electron or a positron ($m_0$) is 0.511 MeV. Therefore, in order for pair production to occur, the minimum energy of the photon must be 1.02 MeV. Thus

$$E(e^- + e^+) = h\nu - 2m_0c^2,$$

where $E(e^- + e^+)$ is the sum of the energies of the positron and the electron and $c$ is the velocity of light. However, if pair production is energetically possible for a photon of energy 1.02 MeV or more, it does not necessarily mean that this process will occur. [For example, a $^{60}$Co $\gamma$-ray with an average photon energy of 1.2 MeV has a pair-production probability so low that virtually all the energy absorption process occurs through the Compton process.] For a photon of energy greater than 1.02 MeV, the
1. Photoelectric Effect  
   (Low energy)  

2. Compton Scattering  
   (High energy)  

3. Pair Formation

Figure 1A.6. Interaction of γ-ray photons with matter.
excess energy is shared by the positron and the electron in the form of kinetic energy. Because the process must be considered as a collision between the photon and the nucleus, the nucleus recoils with some momentum, but the energy involved is very small. The positron, with kinetic energy, has a finite probability of combining with an electron leading to its annihilation. In this annihilation process, two photons (each with 0.511 MeV) are created. However, in water, for example, the entire mode of interaction at these photon energies is through the Compton process.

Considering the γ-rays produced from a 60Co source, with an average energy of 1.2MeV, nearly all the interaction is through the production of Compton electrons. Because the scattered γ-rays have a high probability of penetrating the solution without further interaction, the radiation chemical effects are almost entirely due to the ejected Compton electrons. In this respect it is quite similar to the photoelectric effect, because, in this process also, the chemical effects are caused entirely by the ejected electrons. In conclusion, regardless of the mechanism of interaction involving photons, generation of secondary electrons is an essential phenomenon. All the chemical changes in a medium following photon irradiation are attributed to these electrons.

When an ionising particle, e.g. 100keV electron, passes through absorbing matter, energy is not deposited uniformly along its path but in small packages called 'spurs'. Spurs can have different sizes - the average size of a spur is taken to be 4nm in diameter (Chatterjee and Magee, 1985). They may consist of only one excited molecule, or of several radical cations and electrons as well as a number of excited molecules. On average, about 100eV are deposited in a spur. With high energy electrons, the spurs are an average distance of ~100nm apart. With increasing L.E.T. (Linear Energy Transfer - the energy dissipated per unit length of an electron's path) the spurs curve nearer to one another and cylinders of ionisations and excitations are formed. These are termed 'tracks' and with 60Co γ-rays, the average track length is several millimetres (Myers, 1973).

Radiation action occurs over a broad time scale extending from the early physical processes associated with energy absorption to biological effects, e.g. carcinogenesis, which may not become apparent until many years later. Generally, this time scale can be separated into three stages of radiation action: The physical, chemical and biological (Adams and Jameson, 1980), table 1A.1.

The physical stage essentially begins with the passage of a fast electron or high energy photon through the absorbing medium and the resultant transfer of energy.
### TABLE 1A.1
Timescale of radiation action.

<table>
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<tr>
<th>Time (s)</th>
<th>Process occurring</th>
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<tr>
<td><strong>Physical stage</strong></td>
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</tr>
<tr>
<td>$10^{-18}$</td>
<td>Fast particle traverses small atom</td>
</tr>
<tr>
<td>$10^{-16} - 10^{-17}$</td>
<td>Ionization: $\text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^+ + e^-$</td>
</tr>
<tr>
<td>$10^{-15}$</td>
<td>Electronic excitation $\text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^*$</td>
</tr>
<tr>
<td>$10^{-14}$</td>
<td>Ion-molecule reactions, e.g. $\text{H}_2\text{O}^+ + \text{H}_2\text{O} \rightarrow \text{OH} + \text{H}_3\text{O}^+$</td>
</tr>
<tr>
<td>$10^{-14}$</td>
<td>Molecular variations - dissociation of excited states: $\text{H}_2\text{O}^* \rightarrow \text{H} + \text{OH}$</td>
</tr>
<tr>
<td>$10^{-12}$</td>
<td>Hydration of ions $e^- \rightarrow e^-_{\text{aq}}$</td>
</tr>
<tr>
<td><strong>Chemical stage</strong></td>
<td></td>
</tr>
<tr>
<td>$&lt;10^{-12}$</td>
<td>Reactions of $e^-$ before hydration with reactive solutes at high concentration</td>
</tr>
<tr>
<td>$10^{-10}$</td>
<td>Reaction of $e^-_{\text{aq}}$ and other radicals with reactive solute (concentration $\sim 1\text{M}$)</td>
</tr>
<tr>
<td>$&lt;10^{-7}$</td>
<td>Reactions in spur</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>Homogenous distribution of radicals</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>Reaction of $e^-_{\text{aq}}$ and other radicals with reactive solute (concentration $\sim 10^{-7}\text{M}$, i.e. $\sim 0.01\text{ppm}$)</td>
</tr>
<tr>
<td>1</td>
<td>Free-radical reactions largely complete</td>
</tr>
<tr>
<td>$1 - 10^3$</td>
<td>Biochemical processes</td>
</tr>
<tr>
<td><strong>Biological stage</strong></td>
<td></td>
</tr>
<tr>
<td>Hours</td>
<td>Cell division affected in prokaryotic and eukaryotic cells</td>
</tr>
<tr>
<td>Days</td>
<td>Damage to central nervous system and gastrointestinal tract evident</td>
</tr>
<tr>
<td>~1 month</td>
<td>Haemopoietic death</td>
</tr>
<tr>
<td>Several months</td>
<td>Late kidney damage, lung fibrosis</td>
</tr>
<tr>
<td>Years</td>
<td>Carcinogenesis and genetic death</td>
</tr>
</tbody>
</table>

*Taken from Adams and Jameson (1980).*
which leads to the formation of secondary electrons as described. Electronic excitation processes occur in the time scale of $10^{-15}$ s. Changes in molecular configuration caused by vibrational excitation are somewhat slower. Rotational excitation is naturally slower and can extend from about $10^{-12}$ s.

The chemical stage of radiation action extends, in water, from about $10^{-12}$ s. The physical and chemical stages overlap to include reactions of the electrons before they are solvated, the solvated electron being one of the most fundamental and important species in free radical chemistry. Early in the chemical stage the free radical products of water radiolysis, $e_{aq}$, H and OH are not homogeneously distributed throughout the medium. They are formed in the region of the tracks of the ionising particle before diffusing into the bulk solution. Homogeneous distribution is not achieved until ~$10^{-8}$ to $10^{-7}$ seconds after the initial ionising event. Reactions of these radicals with a given solute occur over a time scale that is dependent on concentration of solute. Average times decrease with increasing solute concentration. Most reactions are over in less than one second.

The biological stage of radiation action begins where cell division is affected, thus causing damage at the cellular level. The time scale for such effects can range from ~1 hour or so for many bacterial systems to several days for some mammalian cells. For organised tissues the response to radiation damage can cover a large time scale, for example, damage to the central nervous system can appear after a few days whereas genetic changes or cancer-induction may not become observable until many years later.

1A.4 'DIRECT' AND 'INDIRECT' RADIATION DAMAGE TO DNA

Water accounts for approximately 75% of the weight of most cells. Thus, three-quarters of the ionisations or excitations generated within the cell occur in the water molecules. The subsequent reaction pathways of the ionised water molecules are crucial in determining the type of radiation damage to DNA that might take place. These pathways, which are greatly dependent on environment, are summarised in (1), scheme 1A.2.

In aqueous solution at room temperature the ionised or excited water molecules undergo various reactions, such as those with other water molecules, to form 'primary' products such as H, OH, $e_{aq}$, H$_2$, H$_2$O$_2$ and H$_3$O$^+$. The yields of these products
expressed as G-values (the number of molecules or radicals formed per one hundred eV of absorbed energy) are listed in table 1A.2. The reducing species generated in irradiated 'bulk' water are solvated electrons (ca. 80%), and hydrogen atoms (ca. 20%). The major oxidising species generated are hydroxyl radicals, formed by the ion-molecule reaction shown in scheme 1A.1.

Most biologically important molecules are liable to attack by free radicals. These reactions often involve specific parts of the biomolecule e.g. hydrogen atom abstraction from a thiol group by H' or 'OH radicals, (2) and (3) in scheme 1A.2. Thus, by these and other types of reaction, the radical centre is transferred to the biomolecule. This form of radiation damage, whereby radiolysis products of water react with biomolecules such as DNA, is known as 'indirect' damage.

**SCHEME 1A.2**

\[
\begin{align*}
\text{H}_2\text{O} & \overset{\gamma}{\longrightarrow} \text{H}^+ + \cdot \text{OH} + \text{e}_{\text{aq}}^- + \text{H}_2 + \text{H}_2\text{O}_2 + \text{H}_3\text{O}^+ \\
\text{RS-H} + \cdot \text{H}^+ & \longrightarrow \text{RS}^- + \text{H}_2 \\
\text{RS-H} + \cdot \text{OH} & \longrightarrow \text{RS}^- + \text{H}_2\text{O}
\end{align*}
\]

It is generally agreed that the most lethal lesion for DNA is the double-strand break (Hutchinson, 1978; Radford, 1985; 1986). Radford (1986) observed a linear relationship between DSBs and induction of lethal lesions, suggesting that DSBs are the major cause of cell death. Thus, numerous studies involving indirect damage to DNA have attempted to probe the mechanism of strand breakage caused by radicals produced by water radiolysis. Studies on the end-groups of broken strands has identified sugars oxidised at C4' (Beesk et al., 1978). This suggests that hydrogen atom abstraction by 'OH at C4' may be a primary reaction. However, it has also been shown that about 80% of 'OH radicals react with the DNA bases (Hoard et al., 1974). Indeed, studies on irradiated solutions of polyuridylic acid (poly [U]) have demonstrated that, in order to account for the number of strand breaks observed, 'OH
attack at the uracil base must lead to strand breaks via radical transfer from the base to
the sugar moiety (Lemaire et al., 1984).

### TABLE 1A.2
The radiolysis of water.

<table>
<thead>
<tr>
<th>Species</th>
<th>( \varepsilon_{ai} )</th>
<th>H'</th>
<th>OH</th>
<th>( \mathrm{H}_2\mathrm{O}^+ )</th>
<th>H_2</th>
<th>( \mathrm{H}_2\mathrm{O}_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>G value</td>
<td>2.7</td>
<td>0.55</td>
<td>2.7</td>
<td>2.7</td>
<td>0.45</td>
<td>0.7</td>
</tr>
</tbody>
</table>

These mechanisms are able to explain SSBs but not DSBs. It is possible that
two SSBs in close proximity may give rise to a DSB. It was suggested by Ward (1981)
that two SSBs could be formed close together by multiple radical attacks as a result of
'spurs'. However, in dilute aqueous DNA solutions the probability of a spur
overlapping the DNA molecule is small. Another possibility is that two 'OH radicals,
produced independently, can attack opposite strands of DNA within a few base pairs.
DSBs have been reported to occur when damage arises on opposite strands within ca.
16 base-pairs. For the first possibility to be correct a linear dependence of DSBs with
dose is expected. With the second, this alters to a 'dose squared' dependence. The
actual dose dependance of DSBs is found to be linear up to a dose of 2.4Kg
(Frankenberg-Schwager et al., 1985), suggesting formation is via a single attack.
Schulte-Frohlinde (1986) has suggested that a single 'OH attack at one strand gives rise
to a SSB and a free radical product, which then attacks the opposite strand to form a
DSB. However, no mechanism was postulated.

When an aqueous solution of DNA is concentrated, such that all the water
molecules are involved in solvation of the DNA molecule, the ionised water molecules
follow a different pathway. Instead of undergoing reactions to from 'OH, H' etc., the
positive 'holes' (\( \mathrm{H}_2\mathrm{O}^+ \)) and electrons migrate to the DNA where they react. The
transfer of electrons to DNA is often referred to as 'dry charge transfer' and results in
the formation of electron-gain and electron-loss species at the bases. This form of
radiation damage to DNA is known as 'direct' damage and forms the basis of the
present study. The most common technique for studying direct damage is electron
paramagnetic resonance spectroscopy (EPR). This technique, which is concerned with the detection of species with unpaired electrons, is described in appendix A. The present study uses EPR to investigate direct damage to frozen aqueous solutions of DNA. It uses model compounds, such as bases, nucleotides and nucleosides, in a variety of low-temperature matrices to assist in the elucidation of the reaction mechanisms involved. The frozen aqueous DNA system is discussed in more detail in chapter 5.
Part B

The pyrimidine $\pi^*$-anion radicals
1B.1 INTRODUCTION

The radiolysis of nucleic acid bases (thymine, cytosine, uracil, adenine and guanine), their nucleosides and nucleotides has received much attention due to the fact that these compounds are constituents of RNA and DNA. Knowledge obtained from the studies on these irradiated subunits is extremely relevant to, and has enhanced the understanding of, the more complex nucleic acids. Over the years a number of excellent reviews have appeared upon the subject of the radiolysis of purines and pyrimidines both in the solid state (Bernhard, 1981; Hüttermann, 1982) and in the liquid phase (von Sonntag and Schuchmann, 1986; von Sonntag, 1987; Steenken, 1989).

In the solid-state X- or γ-irradiation of purine or pyrimidine bases, nucleosides or nucleotides produces radical species which are a result of either electron-loss or electron-gain centres from the bases themselves. The electron-loss centre has only one electron in the highest occupied molecular orbital (HOMO). For the electron-gain centre the electron is accepted into the lowest unoccupied molecular orbital (LUMO). These radicals are termed π-cations and π*-anions to indicate that the unpaired electron occupies orbitals with π-symmetry. This is summarized in scheme 1B.1 where MH is a purine or pyrimidine base and $e_m^{-}$ a mobile electron.

**SCHEME 1B.1**

\[
\begin{align*}
\text{MH} & \xrightarrow{\gamma} \text{MH}^+ + e_m^- \\
\text{MH} + e_m^- & \rightarrow \text{MH}^{-}
\end{align*}
\]

Due to the fact that nucleic acid bases can undergo gain or loss of a proton at heterocyclic groups (such as ring nitrogen atoms or carbonyl groups) upon variation of...
environmental pH, the base concerned may be charged prior to irradiation. Thus electron-gain or -loss may lead, for example, to neutral radicals. Furthermore, those radicals charged after irradiation may quickly dissociate or pick-up protons to gain neutrality. Neither change tends to involve positions of relevant spin density in the π-cation or π*-anion and thus it is extremely difficult to detect by EPR spectroscopy. It should be noted that, regardless of the protonation state, these radicals are still often termed π-cations and π*-anions. The present study is concerned only with the π*-anions, their subsequent reactions on warming, and their relevance to the irradiation of RNA and DNA in the solid state.

The π*-anions of the pyrimidine bases have the unpaired electron residing primarily at the C6, C4 and C2 positions (Baudet et al., 1962). The position of highest spin population is that of C6. Bernhard (1981), in an excellent review, suggested that an excess of negative charge is expected at the N3 and O2 positions for cytosine and at the O4 and O2 positions for uracil/thymine. On the basis of the suggestion that protons are attracted to positions that have undergone the greatest increase in negative charge he lists all the possible protonation reactions of the π*-anions of thymine, cytosine and uracil at different pH values, both prior to and after irradiation.

SCHEME 1B.2

Finally, in cases where the substituent X, at C5, is β to the main site of unpaired electron-spin density and X − is a good leaving group, anion elimination can occur giving rise to a σ-radical. Examples of such leaving groups are NO2 −, Cl −, Br − and I − (scheme 1B.2).
1B.2 THYMINE π*-ANIONS

1B.2.1 Protonation at heteroatoms

On addition of an electron to the thymine base a radical anion is formed (1B.1).

Since this species may subsequently protonate at a heteroatom site, and thus no longer be pristine, it is termed a π*-anion. A significant amount of information concerning thymine π*-anions has been obtained. This has been derived mainly from single crystal studies, the use of low-temperature glasses and, more recently, studies in aqueous solution at room temperature, summarized in table 1B.1. Since most of the electron spin density resides at the C6 position of the thymine ring a large hyperfine splitting results. Thus, a broad doublet EPR spectrum is obtained in the solid state with a typical value for $A_{iso}$ of approximately 14G. Due to the width of these EPR lines no other hyperfine splittings are resolved. Protonation of the thymine radical anion at a heteroatom position (e.g. the exocyclic carbonyl oxygen O4) would be expected within the plane of the pyrimidine ring. This would be extremely difficult to detect with EPR spectroscopy since, with the proton orthogonal to the p-orbital in the ring, the hyperfine coupling is expected to be very small.

Kaalhus and Johansen (1973) suggested that protonation of the thymine radical anion could be induced at the O4 position using a low-temperature acidic glass rather than a neutral or basic one. Using EPR they measured an increase in the C6 proton isotropic hyperfine splitting of about 4G. This observation was supported by a suggestion from Bernhard (1981), on the basis of a study by Henriksen and Jones (1971), that protonation of the thymine base occurs in the pH range 0 to 1 prior to
### TABLE 1B.1
EPR Data for Thymine π*-anions

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>HYPERFINE SPLITTINGS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymine</td>
<td>H₂O, R.T.</td>
<td>-</td>
<td>H6 (11.8)</td>
<td>(0.9) Novais and Steenken (1986)</td>
</tr>
<tr>
<td>Thymine</td>
<td>8M NaOD/D₂O + 5mM K₄Fe(CN)₆, 110K</td>
<td>(2.0030)</td>
<td>N1 (1.35)</td>
<td>-</td>
</tr>
<tr>
<td>Thymine</td>
<td>8M NaOD/D₂O, 77K</td>
<td>(2.0040)</td>
<td>CH₃ (0.9)</td>
<td>-</td>
</tr>
<tr>
<td>Thymine</td>
<td>6M NaOH/H₂O, 77K</td>
<td>(2.0037)</td>
<td>H₄⁺ (0.9)</td>
<td>-</td>
</tr>
<tr>
<td>Thymine</td>
<td>Ethylene glycol/water, 77K</td>
<td>(2.0037)</td>
<td>Other (0.9)</td>
<td>-</td>
</tr>
<tr>
<td>Thymine</td>
<td>5M H₂SO₄/H₂O, 77K</td>
<td>(2.0038)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thymine</td>
<td>12M LiCl/D₂O + K₄Fe(CN)₆, 110K</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Thymine</td>
<td>Anhydrous single crystal, 10K</td>
<td></td>
<td>H6 (14.2)</td>
<td>-</td>
</tr>
<tr>
<td>1-methyl-thymine</td>
<td>12M LiCl/H₂O, 4K</td>
<td>(2.0029)</td>
<td>N1 (14.2)</td>
<td>(2.6)</td>
</tr>
<tr>
<td>1-methyl-thymine</td>
<td>Single crystal/H₂O, 10K</td>
<td></td>
<td>CH₃ (13.5)</td>
<td>(2.1)</td>
</tr>
</tbody>
</table>

* Hyperfine splittings are in O; [G] = 10⁷ T. Principal values are separated by commas. Isotropic values are enclosed in parentheses.
  
* Measurement obtained from simulated experimental spectra.
  
* Peak-to-peak doublet splitting (MHz).
  
* Error = ±0.0004
TABLE 1B.1 (continued)*
EPR Data for Thymine π*-anions

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>H6</th>
<th>N1</th>
<th>CH3</th>
<th>H4'</th>
<th>Other</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3-dimethyl-thymine</td>
<td>12M LiCl/H2O, 4K</td>
<td>(2.0029)</td>
<td>(12.25)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Bernhard and Patrzelk (1989)</td>
</tr>
<tr>
<td>Thymidine</td>
<td>8M NaOD/D2O + 5mM K3Fe(CN)6, 110K</td>
<td>(2.0030)</td>
<td>(13.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Sevilla and van Paemel (1972)</td>
</tr>
<tr>
<td>Thymidine</td>
<td>12M LiCl/D2O + K3Fe(CN)6, 110K</td>
<td>-</td>
<td>(14.0)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Sevilla et al. (1972)</td>
</tr>
<tr>
<td>Thymidine</td>
<td>12M LiCl/D2O, 4K</td>
<td>(2.0030)</td>
<td>(12.4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Bernhard and Patrzelk (1989)</td>
</tr>
<tr>
<td>Thymidine</td>
<td>Single crystal/D2O, 4.2K</td>
<td>-</td>
<td>(11.7)</td>
<td>4.6, 19.8, 10.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Box and Budzinski (1975)</td>
</tr>
<tr>
<td>Thymidine</td>
<td>Single crystal/H2O, 10K</td>
<td>-</td>
<td>(11.8)</td>
<td>4.7, 19.9, 10.8</td>
<td>(2.4)</td>
<td>1.3, 3.1, 2.8</td>
<td>(11.8)</td>
<td>(1.9)</td>
</tr>
<tr>
<td>Thymine tetramer (T4)</td>
<td>12M LiCl/H2O, 4K</td>
<td>(2.0030)</td>
<td>(13.4)</td>
<td>(0.9)</td>
<td>(1.3)</td>
<td>-</td>
<td>-</td>
<td>Bernhard and Patrzelk (1989)</td>
</tr>
<tr>
<td>Thymine tetramer (T4)</td>
<td>5M LiCl + 3M HCl/H2O, 4K</td>
<td>(2.0029)</td>
<td>(13.75)</td>
<td>-</td>
<td>-</td>
<td>(2.5)</td>
<td>-</td>
<td>Bernhard and Patrzelk (1989)</td>
</tr>
<tr>
<td>DNA</td>
<td>Fibre, 77K</td>
<td>(2.0033)</td>
<td>(13.5)</td>
<td>(1.3)</td>
<td>(3.6)</td>
<td>-</td>
<td>-</td>
<td>Gräslund et al. (1975)</td>
</tr>
<tr>
<td>DNA</td>
<td>Fibre, 77K</td>
<td>(2.0033)</td>
<td>(13.6)</td>
<td>(1.4)</td>
<td>(3.9)</td>
<td>-</td>
<td>-</td>
<td>Hüttermann et al. (1984)</td>
</tr>
</tbody>
</table>
electron addition, and that subsequent irradiation produces the O4-protonated radical anion. Furthermore, Bernhard and Patrzalek (1989) also discovered an increase in the doublet hyperfine splitting when a neutral 12M LiCl glass containing thymidine was replaced by an acid glass (5M LiCl + 3M HCl). Similarly, when a thymine tetramer (T₄) was used an increase of doublet hyperfine splitting was observed in the acidified lithium chloride glass and attributed to the O4-protonated radical anion (table 1B.1).

**SCHEME 1B.3**

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{H} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2
\end{align*}
\]
Previous authors have demonstrated that a one-electron adduct of thymine can be induced in aqueous solution at room temperature (Scholes, 1963; Loman and Blok, 1968; Scholes and Simic, 1968). This occurs by the rapid reaction ($k_{1B} = 10^9 M^{-1} s^{-1}$) of a solvated electron ($e_{aq}$), formed as a primary product of the radiolysis of water, with the thymine or uracil base (Anbar et al., 1973; Ross, 1975), scheme 1B.3. It was shown that these uracil and thymine radical anions can undergo rapid protonation at both oxygen and carbon (Deeble and von Sonntag, 1984; Deeble et al., 1985). Protonation at oxygen (2) was found to be faster than at carbon (3). Significantly, at neutral pH, deprotonation at oxygen (-2) was found to be about as facile as protonation (the $pK_a$ of 1B.II is about 7.2, Hayon, 1969). Novais and Steenkeen (1986) produced uracil and thymine radical anions in aqueous solution and, using an in situ radiolysis EPR technique, obtained very accurate coupling constants (table 1B.1). Despite the observation of additional hyperfine splittings no assignment to a proton attached to O4 was made, probably because proton exchange is extremely fast on the EPR timescale.

More recently two examples of protonation at the exocyclic carbonyl oxygen atom (O4) have been observed by EPR at 10K. Thymidine and anhydrous thymine single crystals, grown in H2O and irradiated at 10K, derived an extra 12G hyperfine splitting. This splitting, not observed for the crystals grown in D2O (Box and Budzinski, 1975), was attributed to a proton held well out of the plane of the pyrimidine ring (2.1, chapter 2). For thymidine this observation was explained in terms of the way in which the molecules are packed in the native crystal (Young et al., 1969). Finally, these authors found that no O4-protonated species was observed when irradiations were carried out at 65K. They suggested that previous studies on thymine single crystals had failed to detect this species because irradiations had been carried out at 77K or higher.

1B.2.2 Protonation at carbon atoms

One of the first nucleic acid free radicals to be identified was that of the 5,6-dihydrothymine-5-yl radical (1B.III), known as 'TH (Salovey et al., 1963). The EPR spectrum of this radical consists of eight lines which are derived from an overlapping triplet of quartets. Since 'TH has the electron spin density localised at the C5 position the quartet arises from a 20G splitting from the methyl protons at C5, and the triplet from a 40G splitting from two equivalent protons at C6. Its characteristic EPR
spectrum is easily recognised and has allowed TH to be studied more than any other nucleic acid radical.

There are two possible mechanisms of formation for TH in irradiated thymine-containing systems: (i) Direct hydrogen atom addition to C6 and, (ii) protonation of the thymine radical anion (or possibly the π*-anion) at C6. The former was originally suggested by Salovey et al. (1963), whilst Ormerod (1965) demonstrated that the latter was likely to be important in DNA. As pointed out by Bernhard (1981), although the mechanism of formation is fundamentally different in each case, the structures and thus EPR spectra are indistinguishable. It is therefore necessary to consider both mechanisms when TH formation is observed. Hydrogen atom addition reactions involving pyrimidines will be discussed in more detail in section 1B.6.

Protonation of the thymine radical anion at C6 is essentially an irreversible reaction. It has been shown to occur on warming for a variety of thymine-containing systems (table 1B.5) including: thymidine in a sodium hydroxide glass (Holroyd and Glass, 1968), thymine and thymidine in 12M LiCl glasses (Sevilla et al., 1972), thymine and thymidine-5'-monophosphate in aqueous solution at pH 7 (Novais and Steenken, 1986), oriented DNA fibres (Graslund et al., 1975), frozen aqueous thymidine-5'-monophosphate and DNA (Gregoli et al., 1976; 1982). In each case conversion of an EPR doublet to an octet was observed. It is important to note that the doublets concerned may or may not have derived from O4-protonated radical anions. Henriksen and Jones (1971) suggested that the O4 protonated radical anion was produced using an acidic glass. On warming they found a conversion of thymine doublet into an octet, suggesting that if the radical anion is protonated at O4 a conversion to TH can still occur. However, results for single crystal studies suggest
that such a conversion does not occur. The radical anion of thymine is without doubt protonated at O4 in both thymidine and anhydrous thymine single crystals irradiated and observed by EPR spectroscopy at 10K (Sagstuen et al., 1989; Hole et al., 1991; Sagstuen et al., 1992). On warming, however, no conversion to TH (or its O4-protonated equivalent) was observed.

1B.3 CYTOSINE π*-ANIONS

1B.3.1 Protonation at heteroatoms

One-electron addition to cytosine compounds gives rise to π*-anions for either low-temperature glassy matrices or single crystal studies (Dertinger, 1967; Herak and Galogaza, 1969; Lion and van de Vorst, 1971; Sevilla and van Paemel, 1972; Box et al., 1975; Westhof et al., 1975; Flossmann et al., 1976b; Herak et al., 1977; Close and Bernhard, 1979). As for thymine or uracil, the electron spin density lies primarily at C6, C4 and C2 with C6 being the position of highest spin population (Baudet et al., 1962). This yields a broad doublet EPR spectrum in the solid state with a typical value of $A_{iso}$ of ca.14G - summarised in table 1B.2.

The protonation state of the π*-anions, which have been listed in table 1B.2, is in all cases unknown. Bernhard (1981) suggests that N3 is the most likely site of protonation for the cytosine radical anion. He claims that ENDOR studies of cytidine-3'-monophosphate [3'CMP] (Box et al., 1975) and 2'-deoxycytidine-5'-monophosphate [dCMP] (Close and Bernhard, 1979) provide hyperfine tensors that "serve as benchmarks for a π*-anion protonated at N3" (Bernhard, 1981). The claim is made on the basis that the N3 position of the base is protonated in the parent compound.

Similarly, he argues that the N3 position of the cytosine base in cytosine·HCl [C·HCl] crystals (Westhof et al., 1975) has to be protonated and that subsequent electron addition gives rise to a π*-anion.

Lion and van de Vorst (1971) and Sevilla et al., (1972) generated π*-anions using a sodium hydroxide glass (table 1B.2). The latter noted a decrease in the doublet hyperfine splitting upon adding a substituent to N1 for cytosine, uracil and thymine. The doublet hyperfine values obtained in sodium hydroxide glasses were found to correspond closely to those in the single crystal studies of either Box et al., 1975 or
TABLE 1B.2
EPR Data for Cytosine π\(^{-}\)-anions

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>H\textsubscript{6}</th>
<th>Other</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosine</td>
<td>8M NaOD/D(_2)O, 77K</td>
<td>(2.0035)</td>
<td>(23.0)(^b)</td>
<td>-</td>
<td>Lion and van de Vorst (1971)</td>
</tr>
<tr>
<td>Cytosine</td>
<td>8M NaOD/D(_2)O + 5mM K(_4)Fe(CN)(_6), 110K</td>
<td>(2.0030)</td>
<td>(15.5)(^c)</td>
<td>-</td>
<td>Sevilla and van Paemel (1972)</td>
</tr>
<tr>
<td>Cytidine</td>
<td>8M NaOD/D(_2)O + 5mM K(_4)Fe(CN)(_6), 110K</td>
<td>(2.0030)</td>
<td>(13.4)</td>
<td>-</td>
<td>Sevilla and van Paemel (1972)</td>
</tr>
<tr>
<td>Cytosine.H(_2)O</td>
<td>Single crystal, 130K</td>
<td>-</td>
<td>(14.5)</td>
<td>6.0, 22.5, 15.0</td>
<td>Flossmann et al. (1976a)</td>
</tr>
<tr>
<td>Cytosine.H(_2)O</td>
<td>Single crystal, R.T.</td>
<td>-</td>
<td>(9.5)</td>
<td>-</td>
<td>Herak et al. (1977)</td>
</tr>
<tr>
<td>Cytosine.HCl</td>
<td>Single crystal, 77K</td>
<td>-</td>
<td>(16.4)</td>
<td>9.2, 23.8, 16.3</td>
<td>Westhof et al. (1975)</td>
</tr>
<tr>
<td>Cytosine.HCl</td>
<td>Single crystal, R.T.</td>
<td>-</td>
<td>(6.4)</td>
<td>-</td>
<td>Westhof et al. (1975)</td>
</tr>
<tr>
<td>1-methylcytosine</td>
<td>Single crystal, 12K</td>
<td>-</td>
<td>(12.0)</td>
<td>-</td>
<td>Bernhard (1981)</td>
</tr>
<tr>
<td>Cytidine-3'-monophosphate</td>
<td>Single crystal, 4.2K</td>
<td>-</td>
<td>(12.8)</td>
<td>5.2, 21.2, 12.1</td>
<td>Box et al. (1975)</td>
</tr>
<tr>
<td>2'-deoxycytidine-5'-monophosphate</td>
<td>Single crystal, 12K</td>
<td>-</td>
<td>(13.9)</td>
<td>-</td>
<td>Close and Bernhard (1979)</td>
</tr>
</tbody>
</table>

\(^a\) Hyperfine splittings are in G; 1G = 10\(^{-4}\)T. Principal values are separated by commas. Isotropic values are enclosed in parentheses.

\(^b\) Peak-to-peak doublet splitting (MHz).

\(^c\) Warmed and then recooled to 110K.
Close and Bernhard, 1979. Due to the basicity of the glass however, and the fact that a deuterated solvent (i.e. D$_2$O) was used, it is difficult to determine whether or not protonation has taken place.

Studies in the liquid phase have provided an insight into the protonation state of the radical anions of uracil and thymine (Das et al., 1985; Deeble et al., 1985; Novais and Steenken, 1986). Solvated electrons, produced by the radiolysis of water, react rapidly with cytosine and its derivatives (Hayon, 1969; Scholes, 1978; Hissung and von Sonntag, 1979) to give the radical anion of cytosine (IB.IV).

IB.IV

Hissung and von Sonntag (1979), using a pulse conductometric technique, demonstrated that protonation of these electron adducts occurs extremely rapidly with water. The H-adduct so formed has no pK$_a$ below 10.5. They suggested that protonation had most likely occurred at either N3 or O2 but could not substantiate this experimentally. No corresponding EPR studies on the reaction of the solvated electron with cytosine or its derivatives have been carried out, c.f. thymine and uracil (Novais and Steenken, 1986).

In two unique cases, using single crystals of cytosine monohydrate (C:H$_2$O) and cytosine-HCl (C:HCl), isotropic doublet hyperfine splittings of less than 10G were observed (table 1B.2). The π*-anions involved were formed as major products of irradiation at room temperature (Dertinger, 1967; Westhof et al., 1975; Flossmann et al., 1976b; Herak et al., 1977). It was concluded that a radical anion, protonated at the exocyclic carbonyl oxygen atom O2, had been formed. However, this species may well be formed as a result of direct hydrogen atom addition at the carbonyl oxygen, rather than by protonation of the radical anion.
IB.3.2 Protonation at carbon atoms

Summarised in tables 1B.5, 1B.6 and 1B.7 are C5 and C6 H-adducts of thymine, cytosine and uracil. As previously discussed for thymine, the adducts may be formed by two distinct mechanistic pathways, i.e. direct H-atom addition or protonation of the radical anion (π*-anion). The C5 H-adducts (table 1B.7) are most likely to be formed by direct hydrogen atom addition and will be discussed in detail in section 1B.6. Similarly, many of the C6 H-adduct radicals for uracil and cytosine are likely to be formed by direct hydrogen atom addition (table 1B.6). Indeed, from the data available it seems probable that no thermally induced carbon atom protonation of the radical anion (or π*-anion) of cytosine or its derivatives occurs at all.

The C6 H-adduct radical of cytosine (1B.V) consists of an EPR sextet (Σ, i.e. total sweep width, ≈ 120G), derived from coupling to an α-proton at C5 ($A_{iso} ≈ 17G$) and two equivalent β-protons at C6 ($A_{iso} ≈ 50G$). It has been mainly observed in single crystals at room temperature (Hüttermann et al., 1971; Westhof et al., 1975; Flossmann et al., 1976a and 1976b) or at 77K (Rustgi and Box, 1974; Bernhard and Farley, 1976).

![Image](1B.V)

Carbon atom protonation of the radical anion (or π*-anion) of cytosine may take place but studies using low-temperature aqueous sodium hydroxide glasses have failed to observe the C6 H-adduct on warming (Sevilla et al., 1972; Lion and van de Vorst, 1971). Studies in the liquid phase, where the radical anion of cytosine (and the heteroatom protonated radical anion) is known to form, have picked up the C6 H-adduct (Hissung and von Sonntag, 1979). However, it has been suggested that its mechanism of formation is by direct hydrogen atom addition rather than C6 protonation of the radical anion.
1B.4 URACIL $\pi^+$-ANIONS

1B.4.1 Protonation at heteroatoms

One-electron addition to uracil compounds gives rise to $\pi^+$-anions as observed by single crystal studies (Box et al., 1975; Flossmann et al., 1975b; Zehner et al., 1976; Bergene and Vaughan, 1976; Sagstuen, 1980; Radons et al., 1981; Voit and Hüttermann, 1983), low-temperature aqueous sodium hydroxide glasses (Sevilla and van Paemel, 1972; van de Vorst, 1973) and liquid phase studies (Novais and Steenken, 1986). As for most of the $\pi^+$-anions of cytosine and thymine the protonation state is unknown. Similarly, the EPR spectrum of the uracil $\pi^+$-anion consists of a broad doublet in the solid state with a typical value for $A_{iso}$ of ca. 13 G. This is summarised in table 1B.3.

Bernhard (1981) suggested that heteroatom-protonation of the uracil radical anion is likely to occur at the exocyclic carbonyl oxygen atom (O4). He further claimed that the O4 position in uracil parallels N3 of cytosine as a receptor for protons and that the difference in $pK_a$ for the parent compounds (cytosine $pK_a$ ca. 4 and uracil $pK_a < 1$) remains on electron addition. Thus, the cytosine radical anion should protonate at N3 more readily than the uracil radical anion should protonate at O4. In both cases, however, results from solid state studies give no proof that protonation of the respective radical anions takes place at all (tables 1B.2 and 1B.3).

Results obtained from liquid phase studies, whereby a solvated electron adds to uracil (scheme 1B.3), show that heteroatom-protonation of the uracil radical anion (1B.VI) occurs readily ($K = 10^{10} M^{-1}s^{-1}$). Deeble et al. (1985) suggest that protonation

![1B.VI](image-url)
**TABLE 1B.3**

**EPR Data for Uracil π*-anions**

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>HYPERFINE SPLITTINGS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>H6</td>
<td>H5</td>
</tr>
<tr>
<td>Uracil</td>
<td>H₂O, R.T.</td>
<td>(2.0030)</td>
<td>(12.65)</td>
<td>(0.9)</td>
</tr>
<tr>
<td>1-methyluracil</td>
<td>H₂O, R.T.</td>
<td></td>
<td>(12.3)</td>
<td>(1.5)</td>
</tr>
<tr>
<td>Uridine</td>
<td>H₂O, R.T.</td>
<td></td>
<td>(12.3)</td>
<td>(1.98)</td>
</tr>
<tr>
<td>Uridine-5'-monophosphate</td>
<td>H₂O, R.T.</td>
<td></td>
<td>(12.4)</td>
<td>(1.9)</td>
</tr>
<tr>
<td>2'-deoxyuridine-5'-monophosphate</td>
<td>H₂O, R.T.</td>
<td></td>
<td>(12.4)</td>
<td>(2.0)</td>
</tr>
<tr>
<td>Uracil</td>
<td>8M NaOD/D₂O + 5mM K₃Fe(CN)₆, 110K</td>
<td>(2.0030)ᵇ</td>
<td>(15.2)</td>
<td>-</td>
</tr>
<tr>
<td>Uridine</td>
<td>8M NaOD/D₂O + 5mM K₃Fe(CN)₆, 110K</td>
<td>(2.0030)ᵇ</td>
<td>(14.2)</td>
<td>-</td>
</tr>
<tr>
<td>Uracil</td>
<td>8M NaOH/H₂O, 77K</td>
<td></td>
<td>(14.6)</td>
<td>-</td>
</tr>
<tr>
<td>2-thiouracil</td>
<td>8M NaOH/H₂O, 77K</td>
<td></td>
<td>(15.5)</td>
<td>-</td>
</tr>
<tr>
<td>2'-deoxyuridine</td>
<td>8M NaOH/H₂O, 77K</td>
<td></td>
<td>(11.0)</td>
<td>-</td>
</tr>
<tr>
<td>2'-deoxyuridine-5'-monophosphate</td>
<td>8M NaOH/H₂O, 77K</td>
<td></td>
<td>(12.0)</td>
<td>-</td>
</tr>
</tbody>
</table>

ᵃ Hyperfine splittings are in G; 1G = 10⁻⁴T. Principal values are separated by commas. Isotropic values are enclosed in parentheses.
ᵇ Measurement obtained from simulated experimental spectra.
<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>H6</th>
<th>H5</th>
<th>N1</th>
<th>N3</th>
<th>Other</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine-5'-monophosphate (Ba⁺ salt)</td>
<td>Single crystal/H₂O, 4.2K</td>
<td>-</td>
<td>(12.9)</td>
<td>5.8, 21.1, 11.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Box et al. (1975)</td>
</tr>
<tr>
<td>1-methyluracil.HBr</td>
<td>Single crystal/H₂O, 77K</td>
<td>-</td>
<td>(14.7)</td>
<td>7.5, 22.5, 14.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Flossmann et al. (1975b)</td>
</tr>
<tr>
<td>Uracil</td>
<td>Single crystal/H₂O, 300K</td>
<td>-</td>
<td>(6.7)</td>
<td>4.5, 9.5, 6.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Zehner et al. (1976)</td>
</tr>
<tr>
<td>Uracil-β-D-arabinofuranoside</td>
<td>Single crystal/H₂O, 77K</td>
<td>(2.0036)</td>
<td>(13.1)</td>
<td>5.9, 20.5, 13.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Bergene and Vaughan (1976)</td>
</tr>
<tr>
<td>Uridine-5'-monophosphate (Na⁺ salt)</td>
<td>Single crystal/H₂O, 77K</td>
<td>2.0033, 2.0034, 2.0022</td>
<td>(12.8)</td>
<td>5.7, 21.3, 11.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Sagstuen (1980)</td>
</tr>
<tr>
<td>2'-deoxyuridine</td>
<td>Single crystal/H₂O, 8-77K</td>
<td>(2.0026)</td>
<td>(13.9)</td>
<td>8.3, 21.0, 12.4</td>
<td>(1.5)</td>
<td>(1.5)</td>
<td>-</td>
<td>Voit and Hüttermann (1983)</td>
</tr>
<tr>
<td>Uridine-5'-monophosphate (Na⁺ salt)</td>
<td>Single crystal/H₂O, 10-77K</td>
<td>( - )</td>
<td>(14.1)</td>
<td>7.2, 23.1, 11.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Radons et al. (1981)</td>
</tr>
</tbody>
</table>
is likely to occur at O4 although no direct evidence is presented. Novais and Steenken (1986) also detected the uracil π*-anion in solution using EPR spectroscopy (table 1B.3). They found no splitting attributable to a proton at O4. However, as explained earlier, such a proton would be extremely difficult to detect by EPR since proton exchange is probably very fast on the EPR timescale.

1B.4.2 Protonation at carbon atoms

The C5 and C6 H-adducts of uracil (1B.VII and 1B.VIII) generate EPR spectra very similar to those of cytosine. They consist of sextets. The former is derived from coupling to an α proton at C6 (A_a iso ca. 19G) and two equivalent β protons at C5 (A_β iso ca. 32G). The latter is derived from coupling to an α proton at C5 (A_a iso ca. 20G) and two equivalent β protons at C6 (A_β iso ca. 43G) [Novais and Steenken, 1986]. The C5 H-adducts, listed in table 1B.7, are very likely produced by direct hydrogen atom addition. Most of the C6 H-adducts may also be formed by direct hydrogen atom addition.

Studies in the liquid phase have shown that the uracil radical anion can protonate at both heteroatoms (i.e. O4) and carbon atoms (Deeble and von Sonntag, 1984; Deeble et al., 1985). Novais and Steenken (1986) showed that carbon protonation of the radical anion of uracil occurs preferentially at C6. They noted, however, that hydrogen atom addition could occur at both C5 and C6 - although C5 is preferred (scheme 1B.3). Deeble et al., (1985) noted that protonation at O4 is faster than protonation at C6 but, at neutral pH, deprotonation at carbon (pK_a > 10) is very much slower than at oxygen (pK_a ≈ 7).
1B.5 SUBSTITUTED URACIL $\pi^*$-ANIONS

Listed in table 1B.4 are the hyperfine splitting values for a series of $\pi^*$-anions with substituents at various positions. These can be divided roughly into three groups: (i) Uracils with halogen substituents at the C5 position, (ii) uracils with methyl substituents at positions other than C5 and (iii) uracils with a carbonyl oxygen atom replaced by a sulphur atom (i.e. 2-thiouracil).

1B.5.1 2-Thiouracil

Addition of an electron to 2-thiouracil, both in the liquid phase and in low-temperature aqueous sodium hydroxide glasses, gives rise to a $\pi^*$-anion (van de Vorst, 1973; Novais and Steenken, 1986). This is almost identical to that of uracil and, with the majority of the electron residing at the C6 position, a doublet EPR spectrum is observed - broad in the solid state and well resolved in the liquid phase. The protonation state of the $\pi^*$-anion cannot be determined - even in the liquid phase. No evidence has been found in either study for the existence of the C6 carbon-protonated radical anion of 2-thiouracil.

1B.5.2 Methyl substituted uracil compounds

Placing a methyl group at the ring nitrogen (N3) of uracil produces an increase in the isotropic doublet hyperfine splitting of ca. 2-3G (tables 1B.3 and 1B.4). This was observed in the liquid phase by Novais and Steenken (1986). They demonstrated that the $\pi^*$-anions of both uracil and 3-methyluracil are produced on reaction with a solvated electron, but that the methyl group increases the share of electron spin density at C6. As for uracil the protonation state of the $\pi^*$-anion of 3-methyluracil could not be determined.

If a methyl substituent is placed at the C6 position of the uracil ring the EPR spectrum of the $\pi^*$-anion then consists of four lines (at a ratio of 1:3:3:1). This has been shown both in the liquid phase and using low-temperature aqueous sodium hydroxide glasses (Nucifora et al., 1972; Sevilla and van Paemel, 1972; Novais and Steenken, 1986). The quartet derives from strong coupling to the methyl protons and therefore demonstrates the large electron spin density which exists at C6.
### TABLE 1B.4
EPR Data for Substituted uracil $\pi^*$-anions

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>HYPERFINE SPLITTINGS</th>
<th>Other</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-chlorouracil</td>
<td>H$_2$SO$_4$/H$_2$O</td>
<td>-</td>
<td>(15.0)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5-bromouracil</td>
<td>H$_2$SO$_4$/H$_2$O</td>
<td>-</td>
<td>(15.0)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5-fluouracil</td>
<td>H$_2$SO$_4$/H$_2$O</td>
<td>-</td>
<td>(15.0)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-thiouracil</td>
<td>H$_2$O, R.T.</td>
<td>-</td>
<td>(12.85)</td>
<td>(1.65)</td>
<td>(2.6) (1.3) (0.35)</td>
</tr>
<tr>
<td>3-methyluracil</td>
<td>H$_2$O, R.T.</td>
<td>-</td>
<td>(14.95)</td>
<td>(1.8)</td>
<td>(2.5) (0.6) (0.3)</td>
</tr>
<tr>
<td>6-methyluracil</td>
<td>H$_2$O, R.T.</td>
<td>-</td>
<td>(15.6)</td>
<td>(1.25)</td>
<td>(1.88) (0.63)</td>
</tr>
<tr>
<td>1,3-dimethyluracil</td>
<td>8M NaOD/D$_2$O + 5mM K$_4$Fe(CN)$_6$, 110K</td>
<td>(2.0030)$^d$</td>
<td>(12.3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6-methyluracil</td>
<td>8M NaOD/D$_2$O + 5mM K$_4$Fe(CN)$_6$, 110K</td>
<td>(2.0030)$^d$</td>
<td>(15.4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-thiouracil</td>
<td>8M NaOH/H$_2$O, 77K</td>
<td>-</td>
<td>(15.5)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

---

$a$ Hyperfine splittings are in G; 1G = $10^{-4}$T. Principal values are separated by commas. Isotropic values are enclosed in parentheses.

$b$ Assigned to a hyperfine splitting from a nitrogen nucleus.

$c$ Assigned to a hyperfine splitting from a methyl substituent at C6.

$d$ Error = ±0.0004.
1B.5.3 5-halouracil compounds

5-halogen substituted uracil derivatives have been the source of great interest over the years. The reason comes from the enhanced in vivo radiosensitivity of DNA when some of the thymine bases are replaced by one of the group of 5-halouracils. A quantitative correlation between radiosensitivity enhancement and both the number of thymines replaced and the halogen substituent used has been observed (for a review see Szybalski, 1974).

Electron addition to 5-halouracils differs from that to uracil in that orbitals with \( \sigma \) symmetry are also available to accommodate the excess electron. It has been shown by calculation that the energy difference between the \( \sigma^* \) and \( \pi^* \) LUMOs is negligible for 5-bromo derivatives but favours \( \pi^* \)-anion formation for 5-fluoro and 5-chloro substituents and \( \sigma^* \)-anion formation with 5-iodo (for review see Hüttermann, 1982), scheme 1B.4. These calculations support the experiment studies in acidic, neutral,

SCHEME 1B.4

\[
\begin{align*}
\text{H} & \quad \text{O} & \quad \text{X}_1 & \quad \text{H} \\
\text{O} & \quad \text{N} & \quad \text{R} & \quad \text{X}_1 \\
\text{N} & \quad \text{H} & \quad \text{R} & \\
\text{H} & \quad \text{N} & \quad \text{X}_1 & \\
\text{R} & \quad \text{X}_1 & & \\
\end{align*}
\]

\( X_1 = \text{Br, I} \) \( \sigma^* \)-anion

\[
\begin{align*}
\text{H} & \quad \text{O} & \quad \text{X}_2 & \quad \text{H} \\
\text{O} & \quad \text{N} & \quad \text{R} & \quad \text{X}_2 \\
\text{N} & \quad \text{H} & \quad \text{R} & \\
\text{H} & \quad \text{N} & \quad \text{X}_2 & \\
\text{R} & \quad \text{X}_2 & & \\
\end{align*}
\]

\( X_2 = \text{Cl, Br, F} \) \( \pi^* \)-anion
and basic glasses (Sevilla et al., 1974; Riederer et al., 1978; Oloff, 1981). It was discovered that the α*-anion could undergo halide elimination (scheme 1B.2) to form a uracilyl radical. It was then suggested that the enhancement sensitivity of 5-halo-substituted DNA over that of ordinary DNA could be explained in terms of the uracilyl radical. This α-type radical might abstract intramolecularly a hydrogen atom from a neighbouring deoxyribose to yield the base uracil and a sugar radical. The sugar radical could then act as a precursor to a strand break (Zimbrick et al., 1969). Studies in the liquid phase strongly supported this theory (Kuwabara et al., 1981; Basal et al., 1972), however, for single crystals no trace of halide elimination was observed. Indeed, electron attachment to the halouracil base was observed to yield typically a π*-anion and subsequent reactions identical to those starting from the thymine π*-anion (Hüttermann, 1982).

**SCHEME 1B.5**

![Scheme 1B.5](image)
1B.6 HYDROGEN ATOM REACTIONS

As discussed previously, H-adduct radicals of the pyrimidines occur in numerous systems. It is unlikely that they are all formed by protonation of the respective radical anions (or π*-anions). More likely is the possibility that they are produced as a result of reaction between the pyrimidine base and a hydrogen atom. Such an addition has been shown by EPR spectroscopy to occur across the C5-C6 double bond giving rise to either 5-yl or 6-yl radical species (scheme 1B.5).

The hydrogen atoms can be produced in a variety of ways and systems: (i) by pulse radiolysis of aqueous solution at room temperature, (ii) γ- or X-irradiation of low-temperature glasses, (iii) microwave discharge in hydrogen gas and, (iv) excitation of the pyrimidine itself followed by homolytic bond fission.

1B.6.1 Reactions of hydrogen atoms produced by a microwave discharge in hydrogen gas

This technique was used by Holmes et al. (1966, 1967a, 1967b) to produce hydrogen atom adducts to the pyrimidine bases. They discovered that under vacuum, in the absence of the effects of air or water, gaseous hydrogen atoms react readily with aqueous solutions of pyrimidines which have been rapidly freeze-dried. The resulting spectra demonstrated that addition had taken place across the C5-C6 double bond, confirming the earlier findings of Herak and Gordy (1965, 1966). For thymine the now well characterised 5,6-dihydrothymine-5-yl radical (TH) was observed. For uracil and cytosine EPR sextets were observed. These clearly derived from either the C5 or C6 H-adduct, but no differentiation was made between the two. Inspection of the values in table 1B.6 and 1B.7 strongly suggests that hydrogen atom addition to C5 had occurred in this system giving rise to the 6-yl radical.

1B.6.2 Reactions of hydrogen atoms produced by pulse radiolysis of aqueous solutions

The radiolysis of water at low pH gives rise to the formation of hydrogen atoms (see below).
### TABLE 1B.5
EPR Data for Hydrogen addition to C6 in thymine compounds

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>HYPERFINE SPLITTINGS</th>
<th>S.W.</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CH$_3$</td>
<td>H$_6$$^a$</td>
<td>H$_6$$^b$</td>
</tr>
<tr>
<td>Thymine</td>
<td>12M LiCl/H$_2$O +</td>
<td>-</td>
<td>(20.3)</td>
<td>(79.2)$^c$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10mM K$_4$Fe(CN)$_6$, 170K</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymine</td>
<td>6M H$_2$SO$_4$, 160K</td>
<td>-</td>
<td>(19.5)$^d$</td>
<td>(43.1)</td>
<td>(34.1)</td>
</tr>
<tr>
<td>Thymine</td>
<td>6M H$_2$SO$_4$, 110K</td>
<td>( - )</td>
<td>(20.0)</td>
<td>(41.0)</td>
<td>(35.0)</td>
</tr>
<tr>
<td></td>
<td>2.0050, 2.0050, 2.0030</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymine</td>
<td>Anhydrous single crystal, 10K</td>
<td>-</td>
<td>(20.0)</td>
<td>(45.3)</td>
<td>(32.0)</td>
</tr>
<tr>
<td>Thymine monohydrate</td>
<td>Single crystal, R.T.</td>
<td>-</td>
<td>(21.7)</td>
<td>(40.8)</td>
<td>(34.2)</td>
</tr>
<tr>
<td>1-methylthymine</td>
<td>Single crystal, R.T.</td>
<td>-</td>
<td>(19.9)</td>
<td>(41.0)</td>
<td>(36.8)</td>
</tr>
<tr>
<td>1-methylthymine</td>
<td>Single crystal, 133K</td>
<td>-</td>
<td>(19.9)</td>
<td>(43.1)</td>
<td>(37.6)</td>
</tr>
</tbody>
</table>

$^a$ Hyperfine splittings are in G; 1G = $10^{-4}$T. Principal values are separated by commas. Isotropic values are enclosed in parentheses.

$^b$ Spectral Width.

$^c$ H$_6$$^a$ + H$_6$$^b$.

$^d$ Methyl protons treated as inequivalent.
<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>CH₃</th>
<th>H₆ₐ</th>
<th>H₆₀</th>
<th>S.W.⁺</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine</td>
<td>8M NaOH/H₂O + 5mM K₃Fe(CN)₆, 180K</td>
<td></td>
<td>(20.0)</td>
<td>(43.0)</td>
<td>(34.0)</td>
<td>137</td>
<td>Holroyd and Glass (1968)</td>
</tr>
<tr>
<td>Thymidine</td>
<td>8M NaOD/D₂O + 5mM K₃Fe(CN)₆, 200K</td>
<td></td>
<td>(20.0)</td>
<td>(40.0)</td>
<td>(5.7)⁺</td>
<td>112</td>
<td>Holroyd and Glass (1968)</td>
</tr>
<tr>
<td>Thymidine</td>
<td>12M LiCl/H₂O + 10mM K₃Fe(CN)₆, 170K</td>
<td></td>
<td>(20.2)</td>
<td>(75.2)</td>
<td></td>
<td>136</td>
<td>Sevilla et al. (1972)</td>
</tr>
<tr>
<td>Thymidine</td>
<td>Single crystal, 10K</td>
<td></td>
<td>-</td>
<td>(41.8)</td>
<td>(39.5)</td>
<td>-</td>
<td>Hole et al. (1991)</td>
</tr>
<tr>
<td>Thymidine-5'-monophosphate</td>
<td>H₂O, R.T.</td>
<td></td>
<td>(20.4)</td>
<td>(73.2)</td>
<td></td>
<td>-</td>
<td>Novais and Steenken (1986)</td>
</tr>
</tbody>
</table>

⁺ Assigned as a hyperfine splitting to deuterium at C6.
<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>H5</th>
<th>H6,</th>
<th>S.W.</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosine monohydrate</td>
<td>Single crystal, R.T.</td>
<td>-</td>
<td>(16.7)</td>
<td>(33.0)</td>
<td>(49.0)</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.5, 26.5, 15.0</td>
<td></td>
<td></td>
<td>Flossmann et al. (1976a)</td>
</tr>
<tr>
<td>Cytosine.HCl</td>
<td>Single crystal, R.T.</td>
<td>-</td>
<td>(17.2)</td>
<td>(97.6)</td>
<td></td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.2, 26.7, 17.8</td>
<td></td>
<td></td>
<td>Westhof et al. (1975)</td>
</tr>
<tr>
<td>1-methylcytosine</td>
<td>Single crystal, 77K</td>
<td>-</td>
<td>(17.8)</td>
<td>(51.3)</td>
<td>(47.8)</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(46.9)</td>
<td>(37.6)</td>
<td>Rustgi and Box (1974)</td>
</tr>
<tr>
<td>5-methylcytosine</td>
<td>Single crystal, R.T.</td>
<td>-</td>
<td>(19.5)</td>
<td>(53.1)</td>
<td>(48.8)</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(46.9)</td>
<td>(37.6)</td>
<td>Hüttermann et al. (1971)</td>
</tr>
<tr>
<td>2'-deoxy-cytidine.HCl</td>
<td>Single crystal, R.T.</td>
<td>-</td>
<td>(16.7)</td>
<td>(53.1)</td>
<td>(48.8)</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.5, 26.5, 15.0</td>
<td></td>
<td></td>
<td>Flossmann et al. (1976b)</td>
</tr>
<tr>
<td>Uracil</td>
<td>6M H2SO4, 110K</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0035, 2.0035, 2.0015</td>
<td>8.0, 28.0, 18.0</td>
<td></td>
<td></td>
<td>Riederer et al. (1981)</td>
</tr>
<tr>
<td>Uracil</td>
<td>H2O, R.T.</td>
<td>(2.0035)</td>
<td>(19.6)</td>
<td>(33.0)</td>
<td>(42.0)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(86.8)</td>
<td></td>
<td>Novais and Steenken (1986)</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>6M H2SO4, 110K</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0052, 2.0066, 2.0029</td>
<td>18.0, 9.0, 165.0</td>
<td></td>
<td></td>
<td>Riederer et al. (1981)</td>
</tr>
<tr>
<td>5-chlorouracil</td>
<td>6M H2SO4, 110K</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0072, 2.0083, 2.0018</td>
<td>6.0, 5.0, 18.0</td>
<td></td>
<td></td>
<td>Riederer et al. (1981)</td>
</tr>
<tr>
<td>5-bromouracil</td>
<td>6M H2SO4, 110K</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0200, 2.0240, 2.0003</td>
<td>32.0, 18.0, 92.0</td>
<td></td>
<td></td>
<td>Riederer et al. (1981)</td>
</tr>
</tbody>
</table>

* Hyperfine splittings are in GHz = 10^{-9}T. Principal values are separated by commas. Isotropic values are enclosed in parentheses.

b Spectral Width.

c H6, + H6,.

d Assigned as a hyperfine splitting from the protons on the 5-methyl group.
### TABLE 1B.6*(Continued)*

EPR Data for Hydrogen addition to C6 in cytosine and uracil compounds

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>H5</th>
<th>H6&lt;sub&gt;a&lt;/sub&gt;</th>
<th>H6&lt;sub&gt;b&lt;/sub&gt;</th>
<th>S.W.</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-methyluracil.HBr</td>
<td>Single crystal, 77K</td>
<td>-</td>
<td>(16.7)</td>
<td>(95.0)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>113</td>
<td></td>
<td>Flossmann <em>et al.</em> (1975b)</td>
</tr>
<tr>
<td>Uracil arabinoside</td>
<td>Single crystal, R.T.</td>
<td>-</td>
<td>(19.3)</td>
<td>(47.8)</td>
<td>(45.1)</td>
<td>112</td>
<td>Bergene and Vaughan (1976)</td>
</tr>
<tr>
<td>Uridine</td>
<td>Single crystal, 295K</td>
<td>(2.0033)</td>
<td>(18.7)</td>
<td>(51.4)</td>
<td>(37.6)</td>
<td></td>
<td>Sagstuen (1981)</td>
</tr>
<tr>
<td>Uridine-5'-monophosphate</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O, R.T.</td>
<td>( - )</td>
<td>(19.6)</td>
<td>(82.8)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td></td>
<td>Novais and Steenken (1986)</td>
</tr>
<tr>
<td>Uridine-5'-monophosphate</td>
<td>Single crystal, 270K</td>
<td>(2.0035)</td>
<td>(18.8)</td>
<td>(49.0)</td>
<td>(41.0)</td>
<td>-</td>
<td>Sagstuen (1980)</td>
</tr>
<tr>
<td>2'-deoxyuridine-5'-monophosphate</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O, R.T.</td>
<td>( - )</td>
<td>(19.5)</td>
<td>(84.0)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td></td>
<td>Novais and Steenken (1986)</td>
</tr>
<tr>
<td>2'-deoxyuridine</td>
<td>Single crystal, R.T.</td>
<td>(2.0028)</td>
<td>(18.2)</td>
<td>(48.5)</td>
<td>(38.7)</td>
<td>-</td>
<td>Voit and Hüttermann (1983)</td>
</tr>
</tbody>
</table>
### TABLE 1B.7
EPR Data for Hydrogen addition to C5 in thymine, cytosine and uracil compounds

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>HYPERFINE SPLITTINGS</th>
<th>S.W.</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>H₆</td>
<td>H₅a</td>
<td>H₅b</td>
</tr>
<tr>
<td>Thymine</td>
<td>6M H₂SO₄, 110K</td>
<td>( - )</td>
<td>(38.0)c</td>
<td>-</td>
<td>Riederer et al. (1981)</td>
</tr>
<tr>
<td>Thymine</td>
<td>Anhydrous single crystal, 10K</td>
<td>(19.2)</td>
<td>(48.6)c</td>
<td>-</td>
<td>Sagstuen et al. (1992)</td>
</tr>
<tr>
<td>1-methylthymine</td>
<td>Single crystal, 10K</td>
<td>(19.9)</td>
<td>(49.0)c</td>
<td>-</td>
<td>Hole et al. (1991)</td>
</tr>
<tr>
<td>Thymidine</td>
<td>Single crystal, 10K</td>
<td>-</td>
<td>(47.4)c</td>
<td>-</td>
<td>Hole et al. (1991)</td>
</tr>
<tr>
<td>Cytosine</td>
<td>H₂ discharge, R.T.</td>
<td>(18.0)</td>
<td>(64.0)d</td>
<td>-</td>
<td>Holmes et al. (1967b)</td>
</tr>
<tr>
<td>Cytosine monohydrate</td>
<td>Single crystal, R.T.</td>
<td>(18.6)</td>
<td>(74.0)d</td>
<td>93</td>
<td>Flossmann et al. (1976a)</td>
</tr>
<tr>
<td>Cytosine.D₂O</td>
<td>Single crystal, R.T.</td>
<td>(18.6)</td>
<td>(5.7)c, (37.0)</td>
<td>-</td>
<td>Flossmann et al. (1976a)</td>
</tr>
<tr>
<td>1-methylcytosine</td>
<td>Single crystal, 77K</td>
<td>(17.4)</td>
<td>(45.2)</td>
<td>92</td>
<td>Rustgi and Box (1974)</td>
</tr>
<tr>
<td>2'-deoxy-cytidine.HCl</td>
<td>6M H₂SO₄, 110K</td>
<td>(18.1)</td>
<td>(60.0)d</td>
<td>78</td>
<td>Flossmann et al. (1976b)</td>
</tr>
</tbody>
</table>

* Hyperfine splittings are in G; 1G = 10⁻⁴T. Principle values are separated by commas. Isotropic values are enclosed in parentheses.
* Spectral Width.
* Assigned as one proton attached to C5.
* Assigned as a hyperfine splitting to a deuterium atom at C5.
### TABLE 1B.7" (Continued)

**EPR Data for Hydrogen addition to C5 in thymine, cytosine and uracil compounds**

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX</th>
<th>g-VALUE</th>
<th>HYPERFINE SPLITTINGS</th>
<th>S.W.</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td>H₂ discharge, R.T.</td>
<td>-</td>
<td>(19.0)</td>
<td>-</td>
<td>Holmes et al. (1967b)</td>
</tr>
<tr>
<td>Uracil</td>
<td>6M H₂SO₄, 110K</td>
<td>( - )</td>
<td>(49.0)</td>
<td>-</td>
<td>Riederer et al. (1981)</td>
</tr>
<tr>
<td>Uracil</td>
<td>Single crystal, R.T.</td>
<td>( - )</td>
<td>(35.5)</td>
<td>89</td>
<td>Zehner et al. (1976)</td>
</tr>
<tr>
<td>Uracil</td>
<td>H₂O, R.T.</td>
<td>( - )</td>
<td>(32.1)</td>
<td>-</td>
<td>Novais and Steenken (1986)</td>
</tr>
<tr>
<td>1-methyluracil</td>
<td>Single crystal, 77K</td>
<td>-</td>
<td>(19.3)</td>
<td>(70.7)⁴</td>
<td>90</td>
</tr>
<tr>
<td>1-methyluracil.HBr</td>
<td>Single crystal, 77K</td>
<td>-</td>
<td>(18.6)</td>
<td>(66.0)⁴</td>
<td>85</td>
</tr>
<tr>
<td>Uridine-5'-monophosphate</td>
<td>Single crystal, R.T.</td>
<td>( - )</td>
<td>( - )</td>
<td>( - )</td>
<td>-</td>
</tr>
<tr>
<td>2'-deoxyuridine</td>
<td>Single crystal, R.T.</td>
<td>(2.0029)</td>
<td>(18.4)</td>
<td>(32.9)</td>
<td>-</td>
</tr>
</tbody>
</table>
\[ \text{H}^+ + \text{eaq}^- \rightarrow \text{H}^+ \]

These hydrogen atoms add across the C5-C6 double bond of pyrimidines (scheme 1B.5) in a similar way to those produced in the gas phase (for reviews see von Sonntag and Schuchmann, 1986; von Sonntag, 1987). Results for thymine and uracil suggest that a mixture of the two H-adduct radicals is obtained (Das et al., 1985; Deeble et al., 1985). For uracil, 1,3-dimethyluracil, 6-methyluracil and polyuridylic acid 60-87% of the H-adduct radicals formed are the 6-yl species (i.e H-atom addition to C5). In each case the minor product was the 5-yl radical. For uracil, 1,3-dimethylthymine and thymidine 60-73% of the H-adducts formed were the 5-yl radical (i.e. H-atom addition to C6). Thus, the methyl group has an influence on the position of H-atom attack. It should be noted that with 6-methyluracil the percentage of 6-yl radical formation increased from 69 to 87%.

EPR studies in the liquid phase have detected the formation of both the C5 and C6 H-adducts for uracil (Dohrmann and Livingston, 1971; Novais and Steenken, 1986). The values for the C5 H-adduct are given in table 1B.7 and were accurately measured as an 18.9G α-proton splitting and two equivalent 32.1G β-proton splittings. This is very much in accord with the measured H-adduct splittings of Holmes et al. (1967a, 1967b).

1B.6.3 Reactions of hydrogen atoms produced in low-temperature acidic glasses

The formation of trapped hydrogen atoms upon X- or γ-irradiation of low-temperature acidic glasses (such as H₂SO₄) is well known (Dainton and Jones, 1965; Schulte-Frohlinde and Vacek, 1968; Henriksen and Jones, 1971). These are formed by the reaction of a mobile electron with H⁺ (see below).

\[ \text{H}^+ + \text{em}^- \rightarrow \text{H}^+ \]

In addition to the trapped hydrogen atom signals, which give rise to an EPR doublet (\( \Delta g = 508G \)), a prominent signal, usually assigned to \( \text{SO}_4^{2-} \), is observed (Moorthy and Weiss, 1965). This signal can be avoided by photolysing a H₂SO₄ glass containing
Fe$^{3+}$ ions. Fe$^{3+}$ forms an excited state upon photolysis ($hv > 250$ nm) and an electron is transferred to the surrounding water shell where it reacts with H$^+$ (Moan et al., 1979).

On warming these acidic glasses in the presence of a pyrimidine base the trapped hydrogen atom signal disappears and H-adducts are formed (Riederer et al., 1981). Hydrogen atom addition to uracil, thymine and 5-halouracils occurs at both C5 and C6. The hyperfine splitting values for these adducts are given in tables 1B.6 and 1B.7. The values for the H-adducts of uracil are similar to those obtained in other studies. The C5 H-adduct of thymine however, consisting of a quartet derived from coupling to one $\alpha$-proton at C6 ($A_{iso} \approx 19$ G), and one $\beta$-proton at C5 ($A_{iso} \approx 38$ G), is only observed otherwise in single crystals.

**1B.6.4 Reactions of hydrogen atoms produced by homolytic bond fission**

Homolytic bond fission through an excited state was the method by which hydrogen atoms were produced in the gaseous studies of Herak and Gordy (1965) and Holmes et al. (1966). This section is concerned with homolytic bond fission of a C-H bond in single crystal studies or polycrystalline samples. Fission of the C-H bond should result in the production of two radical species, a hydrogen atom and a carbon-centred radical (scheme 1B.6). It is often difficult to determine experimentally whether radicals are produced by this mechanism or by that of direct ionisation.

**SCHEME 1B.6**
For thymine or its derivatives excitation is believed to be followed by homolytic bond fission at the C5 methyl group (scheme 1B.6) and that the resulting hydrogen atom then adds across the C5-C6 double bond (scheme 1B.5). In single crystal studies the methylene radical species (1B.IX), the 5-yl and the 6-yl radicals have all been detected for thymine, 1-methylthymine and thymidine (Hole et al., 1991; Sagstuen et al., 1992). Since the 6-yl radical has only previously been observed by hydrogen atom addition and since protonation of the radical anion (or \( \pi^2 \)-anion) has been shown to give rise to the 5-yl radical exclusively it seems likely that bond fission has indeed occurred - although it must be noted that the H-adducts formed in each case are minor products.

For cytosine, single crystal studies suggest that homolytic bond fission occurs at the N1-H bond and that the resulting hydrogen atom attaches itself to the C5 position (Cook et al., 1967). It was also tentatively suggested by Dertinger (1967) that the hydrogen atoms add to the O2 carbonyl oxygen. Single crystal studies for cytosine or uracil methylated at the N1 site seem to show that homolytic bond fission takes place at this methyl group. The hydrogen atom then adds to C5 only for 1-methyluracil and 1-methylcytosine but to both C5 and C6 for 1-methyluracil-HBr. It is interesting to note that for a single crystal of 2dC-HCl the C5 H-adduct was observed but no assignment to the site of homolytic bond fission was made.

Flossmann et al. (1976a), using irradiated single crystals of 1-methyluracil, 1-methyluracil-HBr, cytosine-H\(_2\)O and 2'-deoxyctydine-HCl discovered that light (\( \lambda > 400\text{nm} \)) induces a conversion of the C5 H-adduct into the C6 H-adduct. They also found that in some crystals heat could induce a transition in the opposite direction. It was possible for them to monitor such a conversion by using the hyperfine splittings and the relative intensities of the H-adducts (C5 H-adduct is roughly 1:2:1:1:2:1 whereas the C6 H-adduct is 1:1:2:2:1:1).
Chapter Two

The generation of Thymine π*-anions in low-temperature matrices
2.1 INTRODUCTION

Over the years numerous studies concerning the electron-loss centres of pyrimidine nucleobases, nucleosides and nucleotides have been undertaken (see chapter 1B). Although a great deal of information has been obtained through these studies, the exact nature of the species formed is, in most cases, still in doubt (i.e. its protonation state or its site of protonation are unknown). It is for this reason that a detailed study of the one-electron adducts of the pyrimidine bases and derivatives thereof using various low-temperature glassy matrices has been carried out. In chapter 2 the one-electron adducts of thymine derivatives in low-temperature aqueous, aqueous alkali-metal halide, aqueous alkali-metal hydroxide and aqueous alcoholic systems are examined. This study is extended to cytosine and uracil derivatives in chapters 3 and 4 respectively. The relevance of these results to the radiation damage of frozen aqueous solutions of DNA is discussed in chapter 5.

2.1.1 The thymine radical anion and the 5,6-dihydrothymine-5-yl radical

Exposure of duplex DNA to the 'direct' effects of ionising radiations, at temperatures of 77K or below, results in two or more free radical species. One of these has been identified as the thymine radical anion (or thymine \( \pi^- \)-anion). This identification is supported by measurements demonstrating the conversion of an EPR doublet into the easily identifiable eight-line spectrum of the 5,6-dihydrothymine-5-yl radical (Ormerod, 1965). The thymine radical anion, formed by electron addition to the thymine base, has most of its electron spin density at the C6 position of the thymine ring. Thus, a doublet EPR spectrum is observed with a typical value for \( A_{iso} \) of approximately 14G.

Table 1B.1 displays literature values for thymine \( \pi^- \)-anions produced in various systems e.g. single crystals, aqueous solution, low-temperature glasses, etc.. With the exception of single crystal studies on thymine (anhydrous) and thymidine (Sagstuen et
al., 1989; Hole et al., 1991; Sagstuen et al., 1992), it is unclear as to whether any of the thymine radical anions are protonated. Such protonation would be expected at one or more heteroatom positions within the pyrimidine ring rather than at a carbon atom site1. Protonation of a heteroatom site, e.g. the exocyclic carbonyl oxygen atom O4, would be extremely difficult to detect with EPR spectroscopy since, under normal circumstances, the added proton will lie in the plane of the pyrimidine ring. With the proton orthogonal to the p-orbital in the ring, and a relatively low spin population at the neighbouring ring atom site, the hyperfine coupling is expected to be small. Consequently, the hyperfine splitting is unresolved in the solid-state spectrum (Bernhard, 1981). Even in the liquid phase, where much better resolution is achieved, such a splitting could be difficult to detect. Uracil and thymine \( \pi^* \)-anions detected by EPR in solution (pH 7 - 11) show no hyperfine splitting attributable to a proton at a heteroatom site (Novais and Steenken, 1986) despite the fact that protonation may well have occurred (Deeble and von Sonntag, 1984; Deeble et al., 1985).

Anhydrous thymine (denoted as \( \text{T_a} \)) and thymidine single crystals grown in \( \text{H}_2\text{O} \), irradiated and observed by EPR at 10K, have been conclusively shown to protonate at O4 (Sagstuen et al., 1989; Hole et al., 1991; Sagstuen et al., 1992). Sagstuen and co-workers discovered an extra hyperfine splitting, ca. 12G, in the EPR spectra and in both cases assigned it to a proton at the O4 position which is held well out of the plane of the pyrimidine ring (2.1, table 1B.1). For thymidine, this observation was explained in terms of the way in which the molecules are packed in the native crystals (Young et al., 1969). For thymine, no O4-protonated species was observed when irradiations were carried out at 65K and they suggested that previous studies on thymine had failed to pick up this species because irradiations had been carried out at 77K or higher.

Several authors have claimed to induce protonation of \( \text{T}^- \) at O4 under acidic conditions. Kaalhus and Johansen (1973) showed the \( \pi^* \)-anion doublet to have a significantly larger hyperfine splitting in an acidic glass than in a neutral or basic one (table 1B.1), which they attributed to O4-protonation of the radical anion. On the basis of a study by Henriksen and Jones (1971), Bernhard (1981) suggested that protonation of the thymine base occurs in the pH range 0 to -1 prior to electron addition, and that

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1 To differentiate between protonation of the \( \pi^* \)-anion at the various sites the following nomenclature has been adopted: \( \text{T}^-\text{(H')} \) represents the thymine radical anion protonated at any heteroatom position, whereas \( \text{TH} \) represents the thymine radical anion specifically protonated at the C6 carbon atom position.
subsequent irradiation produces the O4-protonated radical anion. Furthermore, in a more recent study an increase in doublet hyperfine splitting was observed when a neutral 12M LiCl glass containing thymidine was replaced by an acidic glass (5M LiCl + 3M HCl). This observation was also attributed to protonation of the radical anion at the O4 position in the acidic glass (Bernhard and Patrzalek, 1989).

Protonation of the thymine radical anion at the carbon atom position C6 occurs readily in many systems (table 1B.5) and can be easily monitored by EPR spectroscopy (Graslund et al., 1975; Gregoli et al., 1982; Boon et al., 1984). It is an essentially irreversible reaction with a large activation barrier and results in the very characteristic eight-line spectrum of the 5,6-dihydrothymine-5-yl radical, TH. Holdroyd and Glass (1968) demonstrated that a thymine π*-anion generated in an 8M sodium hydroxide glass can protonate irreversibly at C6 to give TH. They monitored the TH spectrum on warming and suggested the source of the proton gained at C6 to be the surrounding water molecules in the glass. Similar evidence was presented in the studies by Srinivasan et al. (1969) and Verma et al. (1969) using frozen alkaline and alcoholic matrices containing thymine. All these observations supported the view that the thymine π*-anion is the precursor of TH (Ormerod, 1965).

The spectrum of the 5,6-dihydrothymine-5-yl radical is derived from coupling to three equivalent methyl protons β- to the position of electron spin density at C5 and to two seemingly non-equivalent β-protons at C6. In a few cases H-addition to C5 has been observed (table 1B.7). This results in the formation of the 5,6-dihydrothymine-6-yl radical (2.II). The 6-yl species is characterised by one large β-coupling of nearly 50G (due to a proton at C5) and an anisotropic α-coupling, ca. 20G, to a proton at C6.
2.2 EXPERIMENTAL

2.2.1 Materials

Thymine (T), 1-methylthymine (1mT), thymidine (Thd), 5-methyluridine [ribothymidine (SmU)], thymidine-5'-monophosphate (TMP), thymidylyl(3'→5') thymidine (TpT) and 2'-deoxy-D-ribose (2dR) were purchased from Sigma Chemical Company. Thymidine-d6 (2.111) was kindly donated by Dr. J. Cadet (Laboratoires de Chimie, Département de Recherche Fondamentale, Centre d'Études Nucléaires de Grenoble).

High purity LiCl, K$_3$Fe(CN)$_6$, CH$_2$OH, CD$_3$OD, (CH$_2$OH)$_2$, NaCl, HCl, NaOH pellets and NaOD were purchased from Aldrich Chemical Company, and deuterium oxide (99.98%) from Goss Scientific Instruments Ltd. These materials were used without further purification. The water used was purified using a Millipore 'Multi-Q' system. Liquid nitrogen and nitrogen gas were obtained from British Oxygen Company.

2.2.2 Methods

Stock solutions were prepared of aqueous 10M LiCl (H$_2$O and D$_2$O), 5M LiCl + 3M HCl (in H$_2$O), 5M LiCl + 3M DCl (in D$_2$O), 5.5M sodium chloride (in H$_2$O and D$_2$O), 8M NaOH (in H$_2$O), 8M NaOD (in D$_2$O), methanol/water [(CH$_3$OH/H$_2$O and CD$_3$OD/D$_2$O) at a ratio of 9:2 (v/v)], and ethane-1,2-diol/H$_2$O (at a ratio of 1:1).

Aqueous glasses were prepared using substrate concentrations of 50-100mM, unless stated otherwise. Frozen beads were generated by allowing small droplets of a given solution to fall into a reservoir of liquid nitrogen. Frozen aqueous samples of TMP were prepared by cooling, in liquid nitrogen, a Pyrex tube containing ca. 0.3ml of 250mM solution producing a solid cylinder (pellet). Samples were placed into separate irradiation jars and irradiated at 77K, using a Vickrad $^{60}$Co γ-ray source, for a dose of 0.5-1.0Mrad. The irradiation source dose rate started at 0.5Mrad hr$^{-1}$ (October 1987).
and fell to about 0.35Mrad hr\(^{-1}\) (January 1990). Photolyses were undertaken using an Oriel ultra-violet lamp directed through a Pyrex filter (\(\lambda > 300\text{nm}\)).

EPR measurements were carried out on either a Varian E109 or Jeol JES-RE1X X-band spectrometer at a modulation of 100KHz using a quartz glass finger Dewar inserted directly into the cavity. Both spectrometers are interfaced to an Archimedes computer (software supplied by J. A. Brivati, Department of Chemistry, University of Leicester) for spectral accumulation and subsequent manipulation of data. For the Varian a relative g-factor reference was supplied by the use of a 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) free radical signal during each experiment (\(g = 2.0036\)). For the Jeol a relative g-factor was supplied by the use of a manganese oxide sample. This consists of six lines each with a known g-value \([g(3^{rd} \text{ line}) = 2.034; g(4^{th} \text{ line}) = 1.981]\).

Samples were annealed by two different methods. Directly decanting liquid nitrogen from the finger Dewar made it possible to monitor changes in the EPR spectrum as the sample warmed up. Significant changes having occurred, the sample was then re-cooled to 77K and the spectrum re-recorded. To roughly estimate the temperature at which the sample was re-cooled, a thermocouple was placed inside the finger Dewar. The alternative method of warming samples involved the use of a copper-block cryostat, figure 2.1. This simply consisted of a block of copper with two holes drilled into it. One contained a sample holder and the other a thermocouple. Samples were then allowed to warm to a predetermined temperature, which was maintained for seven minutes to allow them to equilibrate, before being re-cooled to 77K and recorded by EPR.
Figure 2.1. Copper block cryostat for annealing samples
<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>H₂O/D₂O</th>
<th>10M LiCl</th>
<th>5M LiCl + 3M HCl</th>
<th>5.5M NaCl</th>
<th>METHANOL</th>
<th>ETHYLENE GLYCOL</th>
<th>8M SODIUM HYDROXIDE</th>
<th>FROZEN AQUEOUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymine (T)</td>
<td>H₂O</td>
<td>24.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>23.5 (11.8)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D₂O</td>
<td>23.4 (12.0)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1-methylthymine (1mT)</td>
<td>H₂O</td>
<td>23.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D₂O</td>
<td>23.0 (11.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thymidine (Thd)</td>
<td>H₂O</td>
<td>23.5</td>
<td>24.4</td>
<td>22.9</td>
<td>25.6</td>
<td>21.3</td>
<td>22.8</td>
<td>24.6</td>
</tr>
<tr>
<td></td>
<td>D₂O</td>
<td>23.1 (11.8)</td>
<td>24.3</td>
<td>22.2 (11.3)</td>
<td>23.8</td>
<td>-</td>
<td>22.3 (11.4)</td>
<td>23.5</td>
</tr>
<tr>
<td>5-methyluridine (5mU)</td>
<td>H₂O</td>
<td>23.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D₂O</td>
<td>23.0 (11.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thymidine-5'-monophosphate (TMP)</td>
<td>H₂O</td>
<td>23.6</td>
<td>-</td>
<td>23.1</td>
<td>-</td>
<td>23.1</td>
<td>23.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D₂O</td>
<td>23.1 (11.8)</td>
<td>-</td>
<td>22.8 (11.7)</td>
<td>-</td>
<td>22.5 (11.5)</td>
<td>23.0</td>
<td>-</td>
</tr>
<tr>
<td>Thymidyl(3'→5')-thymidine (T₃P₃)</td>
<td>H₂O</td>
<td>23.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D₂O</td>
<td>23.1 (11.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thymidine-d6 (Thd-d6)</td>
<td>H₂O</td>
<td>8.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D₂O</td>
<td>7.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.4</td>
<td>-</td>
</tr>
</tbody>
</table>

**a** Two sets of values are used in this table. The main value is a measure of peak-to-peak distances of the outer lines of the doublets (i.e. ΔH); estimated error = ±0.3G. The values in parentheses are C₆ isotopic hyperfine splittings as estimated by simulation of EPR spectra - see text.

**b** 1G = 10⁻⁴T.

**c** Only ΔH values are given.

**d** Measurement taken close to the melting temperature of the glass - see text.

**e** ΔH value corresponds to an unresolved singlet.
2.3 RESULTS AND DISCUSSION

2.3.1 The radiolysis of frozen aqueous solutions of alkali-metal chlorides

It has been established that electron-capture by suitable solutes in alkali-metal halide glasses is far more efficient than electron-loss (Symons, 1981). Thus, in the presence of such solutes electron-loss is confined solely to the glass. Radical species produced as a result of X- or γ-irradiation, or UV-photolysis, of these glassy systems can easily be detected by EPR spectroscopy at low-temperatures. Indeed, studies on low-temperature chloride glasses have identified species such as Cl₂⁻ (Zvi et al., 1969; Ginns and Symons, 1972). Zvi et al. (1969) observed Cl₂⁻ on UV-photolysis of 10M lithium chloride containing a small concentration of K₂S₂O₈. They also went on to show that Br₂⁻ and I₂⁻ are produced using the corresponding 10M lithium bromide and lithium iodide glasses. Ginns and Symons (1972) carried out a thorough investigation of γ-irradiated low-temperature alkali-metal halide glasses using EPR spectroscopy. As well as Cl₂⁻ radicals they observed hydrogen atom adducts to alkali-metals (MH⁺) and a hydroxyl radical adduct (ClOH⁺). In the present study an attempt will be made to establish both the nature of these systems (i.e. whether or not glass formation has taken place) and their suitability for producing one-electron adducts to pyrimidines.

2.3.1.1 Lithium chloride (10M LiCl)

Samples were prepared at different lithium chloride concentrations and, using the procedure described in the experimental section, monitored by EPR spectroscopy at 77K. At relatively low salt concentrations (i.e. 1M) phase separation was detected. This was inferred by the somewhat opaque nature of the samples prior to irradiation and by the observation of hydroxyl radicals in the EPR spectrum. As will be discussed later hydroxyl radicals, which essentially give rise to an anisotropic EPR doublet (chapter 5), are formed within a separate ice-phase and react independently of radicals formed in
the glassy region of the sample. At higher concentrations of lithium chloride (i.e. 10M) no hydroxyl radicals are detected and the sample is visibly 'transparent' prior to irradiation. This suggests that suitable glass formation has occurred at the higher concentration.

Figure 2.2a shows the EPR spectrum generated as a result of irradiation, at 77K, of the 10M LiCl/H₂O glass. Many different radical species are obtained. One of these, consisting of a characteristic doublet with an isotropic hyperfine splitting (Aiso) of ca. 510G, appears to be that of the hydrogen atom (H; nuclear spin, I, = 1/2) which has been trapped within the glass. Changing the solvent from water to deuterium oxide replaces the doublet with an EPR triplet (2Aiso = 156G) corresponding to deuterium atoms (D; I = 1). Both H and D are believed to form by the reaction of mobile electrons (e\textsuperscript{−}), produced by ionisation of H₂O (D₂O) and/or Cl\textsuperscript{−} [(1) and (2) in scheme 2.1], with protons (H\textsubscript{3}O\textsuperscript{+}) [(10) in scheme 2.1].

Following irradiation of the glass a pale blue sample is obtained. This colour has been shown by Riederer \textit{et al.}, (1981) to correspond to electrons trapped within the glass (e\textsuperscript{−}), and its EPR spectrum consists of a singlet at approximately the 'free-spin' value, figure 2.2a. The singlet disappears on photobleaching along with the blue colour of the sample and no other changes in the EPR spectrum are observed. This poses a question as to the fate of the electron once it becomes mobile. One possibility is the further production of hydrogen atoms. However, there is no compelling reason why e\textsuperscript{−} should react with neighbouring protons (H\textsubscript{3}O\textsuperscript{+}) having preferentially trapped within the glass in the first place. Another possibility is the formation of trapped electron pairs (e\textsuperscript{−}; e\textsuperscript{−}). These species would presumably be non-detectable both optically and by EPR, making their identification otherwise extremely difficult.

An additional singlet is also detected in the spectrum of the irradiated glass. The presence of 'satellite' features suggests that this corresponds to the alkali-metal hydrogen- or deuterium-adduct (MH\textsuperscript{+} or MD\textsuperscript{+}), as observed by Ginns and Symons (1972). The hyperfine tensors (A₀ and A₁), given as a result of coupling from the hydrogen atom to the metal ion, are almost identical to those for a hydrogen atom trapped by itself. Thus, it is extremely difficult to distinguish between H\textsuperscript{−} and LiH\textsuperscript{+}.

The adduct, therefore, is perhaps better pictured as a trapped hydrogen atom weakly interacting with a neighbouring Li\textsuperscript{+} ion (i.e. Li\textsuperscript{+}; H\textsuperscript{−}), and similarly for the deuterium-adduct (Li\textsuperscript{+}; D\textsubscript{−}).
Figure 2.2a. EPR spectrum of a γ-irradiated frozen aqueous solution of 10M lithium chloride, recorded at 77K, showing features attributable to both electron-loss and electron-capture.
Figure 2.2b.
EPR spectrum of a γ-irradiated frozen aqueous solution of 10M lithium chloride containing 100mM thymidine, recorded at 77K.
Thus far only electron-capture species (e', H/D' and Li+... H/Li+... D') have been considered. At least one and probably two other radicals are formed as a result of electron-loss. The major product of electron-loss is that of the Cl2− radical. Its EPR spectrum consists of two sets (parallel and perpendicular) of seven groups of lines (Zvi et al., 1969; Ginns and Symons, 1972), figure 2.2b, arising from coupling to two equivalent chlorine nuclei. Each group of lines further consists of hyperfine features due to combinations of the two chlorine isotopes 35Cl and 37Cl. Marked on figure 2.2b are the parallel features (0, ±1, ±2, ±3) associated with the (35Cl−37Cl)− species, A, ca. 100G. As suggested by Ginns and Symons (1972), the corresponding perpendicular features appear to be superimposed upon those of another radical signal, possibly that of ClOH−.

\[ \text{H}_2\text{O} \xrightarrow{\gamma} \text{H}_2\text{O}^+ + e_m^- \]  \hspace{1em} (1)
\[ \text{Cl}^- \xrightarrow{\gamma} \text{Cl}^+ + e_m^- \]  \hspace{1em} (2)

\[ \text{H}_2\text{O}^+ + \text{H}_2\text{O} \rightarrow \text{H}_2\text{O} + \text{H}_2\text{O}^+ \]  \hspace{1em} (3)
\[ \text{H}_2\text{O}^+ + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \cdot\text{OH} \]  \hspace{1em} (4)
\[ \cdot\text{OH} + \text{Cl}^- \rightarrow \text{ClO}\text{H}^- \]  \hspace{1em} (5)
\[ \text{ClO}\text{H}^- + \text{H}^+ \rightarrow \text{Cl}^- + \text{H}_2\text{O} \]  \hspace{1em} (6)
\[ \text{Cl}^- + \text{Cl}^+ \rightarrow \text{Cl}_2^- \]  \hspace{1em} (7)
\[ \text{Cl}^- + \text{ClO}\text{H}^- \rightarrow \text{Cl}_2^- + \cdot\text{OH} \]  \hspace{1em} (8)

\[ e_m^- + \text{cavity} \rightarrow e^+ \]  \hspace{1em} (9)
\[ \text{H}_2\text{O}^+ + e_m^- \rightarrow \text{H}^+ + \text{H}_2\text{O} \]  \hspace{1em} (10)
\[ \text{Li}^+ + \text{H}^+ \rightarrow (\text{Li}...\text{H})^+ \]  \hspace{1em} (11)
\[ \text{H}^+ + \text{H}^+ \rightarrow \text{H}_2 \]  \hspace{1em} (12)
The mechanism of formation of all these radicals is summarised in scheme 2.1. Damage to water or chloride ions involves electron-loss, (1) and (2) respectively, followed by electron trapping (hydration) (9), and reaction to give ultimately hydroxyl radicals, (3) and (4), and hydrogen atoms (10). Hydroxyl radicals, which may also be present in the EPR spectrum, can react with a chloride ion to give ClO\textsuperscript{-} (5). Hydrogen atoms are either trapped within the glass or form adducts with neighbouring Li\textsuperscript{+} ions (Li\textsuperscript{+}... H) (11). Chlorine atoms, formed by direct ionisation (2) or reaction between ClO\textsuperscript{-} and H\textsuperscript{+} (6), proceed to form Cl\textsubscript{2}\textsuperscript{-} (7). Cl\textsubscript{2}\textsuperscript{-} may also be formed as a result of a reaction between ClO\textsuperscript{-} and a chloride ion (8).

2.3.1.2 Sodium chloride (5.5M NaCl)

Unlike lithium chloride, at all concentrations of sodium chloride (right up to and including saturation point) white, opaque beads are produced on freezing. This is indicative of some degree of ice formation and phase separation within the system. The EPR spectrum obtained as a result of irradiation of a frozen solution of 5.5M sodium chloride is shown in figure 2.3. Many of the features seen previously for lithium chloride are also observed in this system. The hydrogen atom (or alkali-metal hydrogen-adduct) lines are clearly visible (A\textsubscript{iso} ca. 510G). It should be noted, however, that the singlet associated with Na\textsuperscript{+}... H, presumably present, is masked by a strong doublet signal. This doublet is believed to be due either to hydroxyl radicals, or to the radical species ClOH\textsuperscript{-}, or possibly both. On irradiation of the corresponding deuterium oxide system the hydrogen atom lines are replaced by those of deuterium (D\textsuperscript{+}).

2.3.2 The one-electron adducts of thymine and its derivatives in frozen aqueous solutions of lithium chloride

2.3.2.1 Lithium chloride (10M LiCl)

As demonstrated earlier, 10M LiCl/H\textsubscript{2}O forms a good glass on freezing. Gamma-irradiation produces mobile electrons (E\textsubscript{m}) which either react with neighbouring H\textsubscript{2}O\textsuperscript{+} to produce hydrogen atoms or become trapped within the glass. However, the addition of a suitable solute can provide an alternative electron-trap centre for these electrons. This is shown in figure 2.2b where the EPR spectrum of a γ-
Figure 2.3. EPR spectrum of a γ-irradiated frozen aqueous solution of 5.5M sodium chloride, recorded at 77K, showing features attributable to both electron-loss and electron-capture.
irradiated frozen solution of 10M LiCl/H2O containing 100mM thymidine has been recorded. Features for radicals assigned to ionisation of the solvent (e.g. Cl2−) are still present, but those associated with electron capture have now been replaced by a doublet (ΔH = 23.5G, Aiso ca. 12G). Furthermore, the signal associated with H− has been replaced by the outer lines of the 5,6-dihydrothymine-5-yl radical (TH). This is all better illustrated in figure 2.4a where thymine is used as the solute and the spectrum recorded over a narrower scan range (200G). Replacing thymidine with thymine - which contains no sugar moiety - also yields a doublet (ΔH = 24.3G). Therefore, provided the observed doublets are those of the electron-adducts, it seems reasonable to suggest that the electron attaches itself directly to the thymine base and not the sugar moiety. More evidence for this assertion is provided when thymine is replaced by 2'-deoxy-D-ribose (which contains no base moiety). The very weak signal observed for the electron-capture species is completely different from the strong signal associated with capture at the base.

By replacing water with deuterium oxide a somewhat sharper and better resolved thymine doublet is obtained, figure 2.4b. Indeed, using other thymine derivatives such as TMP, 5Mu and TpT, similar doublets in either H2O or D2O are obtained. Although the deuterium glass brings no major changes to the thymine doublet the outer lines of TH are replaced by those of the corresponding D-adduct, TD (2.1V). Thus, since there is a concurrent loss of deuterium atom (D') triplet it seems reasonable to suggest that, under these circumstances, both TH and TD are formed by hydrogen/deuterium atom addition respectively to the thymine base. Indeed, addition at C6 would give such radicals and has been shown to occur in glasses where H'/D' atoms are produced (Riederer et al., 1981).

All these thymine doublets are believed to derive from addition of an electron to the thymine base and proof of this is obtained by the use of a powerful electron scavenger. On addition of potassium ferricyanide [K3Fe(CN)6] to any of these systems formation of the EPR doublet is completely suppressed. The one-electron adduct is, in fact, the radical anion of thymine and, as explained in chapter 1B, a large doublet hyperfine splitting arises from coupling to a proton at C6 - this being the major site of electron spin population. Furthermore, due to the lack of other hyperfine structure within the doublet it is impossible to deduce whether or not the radical anion is protonated. Many authors refer to these species as π*-anions regardless of their

ΔH is defined as the splitting between the outer lines of the doublet (γ1, figure 2.4a).
Figure 2.4a.

ESR spectrum of a γ-irradiated frozen aqueous solution of 10M lithium chloride containing 100mM thymine, recorded at 77K, showing a doublet assigned to the thymine x^*-anion and features attributed to the thymine C6-H-adduct radical T6H. 

\[ y_1 = \Delta H \] (i.e. the magnetic field separation of the doublet peaks).
Figure 2.4b. EPR spectrum of a γ-irradiated frozen solution of 10M lithium chloride (in D2O) containing 100mM thymine, recorded at 77K, showing a doublet assigned to the thymine π*-anion and features attributed to the thymine C6 D-adduct radical 'TD.'
protonation state. In the present study the term π*-anion will be used to refer to radical anions of unknown protonation state. Given in table 2.1 are a set of A-values, based upon simulation, for π*-anions obtained in the present study for various glassy systems.

On warming to 150K, using the method described in the experimental section, EPR features attributable to ionisation of the solvent (i.e. Cl− and ClOH−) are removed, figure 2.5a. These are replaced by the outer lines of the 'TH radical and an additional low-field feature, marked β on figure 2.5a. Further warming - up to the melting temperature of the glass - induces a decay of the thymine π*-anion and an increase in TH. Previously, it has been suggested that 'TH is formed by protonation at C6 of the thymine π*-anion and that the proton is derived from the surrounding solvation water (Holroyd and Glass, 1968). This is supported in the present study when H2O is replaced by D2O. On warming the D2O system to 150K and above an increase of the C6 D-adduct radical (TD) is detected, figure 2.5b. Although it is possible for such a protonation to occur from a neighbouring heteroatom site it seems likely that TH (or TD) is formed by protonation (or deuteronation) of the π*-anion directly from the solvent.

Previous studies have shown that photobleaching glassy systems in which DNA base radical anions have been obtained induces the formation of hydrogen adduct radicals (Holroyd and Glass, 1968; Lion and van de Vorst, 1971; Sevilla et al., 1976). For thymine, this has been demonstrated by the EPR and UV studies of Lion and van de Vorst (1971) where the π*-anion is gradually replaced by 'TH. This observation is confirmed in the current study using a lithium chloride glass containing TMP and which has been photolyzed (λ>300nm) for five minutes.

2.3.2.2 Acidified lithium chloride (5M LiCl + 3M HCl)

It has been suggested by previous authors that the thymine π*-anions generated in many of the low-temperature glasses are pristine (Bernhard, 1981; Bernhard and Patrzalek, 1989). Indeed, on the basis of a study by Kaalhus and Johansen (1973) Bernhard (1981) claimed that protonation of the π*-anion had been induced by the use of a 6M H2SO4/H2O glass. This claim was supported further by results using a 5M LiCl/3M HCl/H2O glass (Bernhard and Patrzalek, 1989), where the linewidth of the thymine doublet was found to increase in the acidified glass relative to that of the 12M
Figure 2.5a. EPR spectrum of a γ-irradiated frozen aqueous solution of 10M lithium chloride containing 100mM thymidine, warmed to 150K, recorded at 77K, showing the growth of a feature marked β.
Figure 2.5b. EPR spectrum of a γ-irradiated frozen solution of 10M lithium chloride (in D₂O) containing 100mM TMP, warmed to 150K and recorded at 77K.
LiCl glass. The increase was attributed to an extra splitting from a proton attached to O4.

In the present study irradiation of 5M LiCl/3M HCl/H$_2$O, in the absence of solute, produces a spectrum which exhibits the expected solvent radical signals, i.e. Cl$_2^-$ and ClOH$^-$, and a singlet indicative of trapped electrons, figure 2.6a. In the presence of thymidine or TMP a broad doublet replaces this singlet figure 2.6b. There can be little doubt that a significant linewidth increase has occurred on changing the solvent from 10M LiCl/H$_2$O to 5M LiCl/3M HCl/H$_2$O. Furthermore, an improvement in resolution and a reduction in the linewidth are detected in the corresponding deuterated solvent system (5M LiCl/3M DCl/D$_2$O). These results certainly support the experimental findings of Bernhard and Patrzalek but they are not overwhelming proof of the pristine nature of the radical anion in the non-acidified lithium chloride glass.

2.3.3 The one-electron adduct of TMP in frozen aqueous solutions of sodium chloride

Although some degree of phase separation seems to occur on freezing aqueous solutions of 5.5M sodium chloride, similar results to those acquired for thymine derivatives in 10M lithium chloride are obtained, figure 2.7a. Features attributable to electron-capture centres within the solvent are replaced by the expected thymine $\pi^*$-anion doublet ($A_{iso}$ ca. 12G, table 2.1), and the thymine C6 H-adduct radical (or D-adduct in the corresponding D$_2$O glass). It should be noted that the EPR doublet is less well resolved for TMP in a sodium chloride glass than in the lithium chloride glass. This may well be due, at least in part, to the superimposition upon this doublet of a signal from radicals produced by ionisation of the solvent medium (i.e. 'OH/ClOH$^-$').

On annealing, a loss of $\pi^*$-anion and a concurrent gain of TH are observed, figures 2.7b and 2.7c. At ca. 175K, when all the solvent features have been removed, a new feature is revealed. Previously marked $\beta$, figure 2.5a, it is possible that this feature belongs to a quintet pattern, figures 2.7d and 2.7e. Due to the greater stability of the sodium chloride glass the annealing process can be continued well beyond 170K. The quintet, which is also observed for $\gamma$-irradiated frozen aqueous TMP, possibly derives from the thymine C6 OH-addition radical (2.V). If OH-addition to C5 of the thymine base occurred in such a way as to 'fix' the proton at C5, thereby giving rise to a
Figure 2.6a. EPR spectrum of a $\gamma$-irradiated frozen aqueous acidified lithium chloride glass (5M LiCl + 3M HCl), recorded at 77K, showing features attributable to electron-loss and electron-capture (c.f. figure 2.2a).
Figure 2.6b. EPR spectrum of a γ-irradiated frozen aqueous acidified lithium chloride glass (0.5 M LiCl + 3 M HCl) containing 100 mM TMP, recorded at 77 K, showing a broad doublet assigned to the thymine π•-cation.
Figure 2.7. EPR spectra of a γ-irradiated frozen aqueous solution of 5.5M sodium chloride containing 100mM TMP, (a) at 77K, (b) warmed to 150K, (c) warmed to 175K. All spectra recorded at 77K.
Figure 2.7 cont. EPR spectra of a γ-irradiated frozen aqueous solution of 5.5M sodium chloride containing 100mM TMP, (d) warmed to 220K and (e) warmed to 240K, showing growth of features assigned to a quintet. All spectra recorded at 77K.
hyperfine splitting of only about 20G (c.f. 40G in TH), then a quintet might arise. The possible consequences of formation of this radical will be discussed fully in chapter 5.

2.3.4 The radiolysis of frozen aqueous solutions of alcohols

2.3.4.1 Methanol

To obtain 'glassy' beads upon the freezing of aqueous solutions of methanol it is necessary to have a high methanol/water ratio (v/v). At ratios where more water than

**SCHEME 2.2**

\[
\begin{align*}
H_2O & \xrightarrow{\gamma} H_2O^{++} + e_m^- \\
CH_3OH & \xrightarrow{\gamma} CH_3OH^{++} + e_m^- \\
H_2O^{++} + H_2O & \xrightarrow{} H_2O + H_2O^{++} \\
H_2O^{++} + H_2O & \xrightarrow{} H_3O^+ + 'OH \\
CH_3OH + H_2O^{++} & \xrightarrow{} CH_3OH^{++} + H_2O \\
CH_3OH^{++} + H_2O & \xrightarrow{} CH_3O^- + H_3O^+ \\
CH_3OH^{++} + ROH & \xrightarrow{} CH_3O^- + ROH_2^+ \\
CH_3OH^{++} + H_2O & \xrightarrow{} 'CH_2OH + H_3O^+ \\
CH_3OH^{++} + ROH & \xrightarrow{} 'CH_2OH + ROH_2^+ \\
\end{align*}
\]

\[
\begin{align*}
e_m^- + \text{cavity} & \xrightarrow{} \epsilon_1^- \\
CH_3OH + e_m^- & \xrightarrow{} H^+ + CH_3O^- \\
CH_3OH + e_m^- & \xrightarrow{} 'CH_3 + 'OH \\
H_2O^{++} + e_m^- & \xrightarrow{} H^+ + H_2O \\
\end{align*}
\]

\[
\begin{align*}
CH_3O^- + CH_3OH & \xrightarrow{} CH_3OH + 'CH_2OH \\
\end{align*}
\]
alcohol is present opaque beads are formed - indicative of phase separation. On increasing the alcohol/water ratio transparent beads, suggesting good glass formation, are produced. A suitable methanol/water ratio for such glass formation is 9:2 (v/v).

The radiation chemistry of alcohols has been reported as having two major pathways (Symons and Eastland, 1977). One involves electron-gain and leads ultimately to hydrogen atoms or alkyl radicals. The other, electron-loss, leads to alkoxy radicals. The radiation chemistry of methanol, however, differs somewhat from other alcohols, such as t-butanol, in that carbon-centred radicals are produced as a result of electron-loss (Rao and Symons, 1973). Scheme 2.2 illustrates possible reactions for both pathways.

No primary radical species (i.e. H₂O⁺ and CH₃OH⁺) are observed by EPR at 77K for the γ-irradiated methanol/water system, figure 2.8a. These radical cations are undoubtedly formed, by ionisation of the parent molecule, (1) and (2), but are not stable and react to give more stable secondary products. The most significant secondary radical species is that of CH₂OH. The spectrum for this radical consists of a triplet characterised by strong coupling to two equivalent α-protons (Aiso, ca. 22G) (Verma et al., 1969). Coupling to the β-proton is weak and yields almost negligible splitting. The radical can be formed from the radical cation CH₃OH⁺ in two distinct ways: (i) Direct deprotonation at the carbon atom (8) and (9), or (ii) deprotonation at the oxygen atom (6) followed by hydrogen abstraction from a neighbouring methanol molecule (14).

Since neither 'OH (characterised by an anisotropic doublet splitting, Riederer et al., 1983) nor CH₃O' (characterised by a large anisotropic g-value, Symons and Eastland, 1977) are present in the spectrum it can be assumed that if formed, by (4) and (6) respectively, both will abstract hydrogen atoms from nearby methanol molecules (14) and (15). It should be noted, however, that there are numerous radical recombination reactions that might also take place. Finally, when CH₃OH is replaced by its fully deuterated analogue CD₃OD, and water by deuterium oxide, the 1:2:1 EPR triplet for 'CH₂OH is replaced by an unresolved singlet. This corresponds to the 'CD₂OD radical which, if resolved, would be expected as a 1:2:3:2:1 quintet, figure 2.8b.
Figure 2.8. EPR spectra of γ-irradiated frozen solutions of (a) CH₃OH/H₂O (9:2 v/v), (b) CD₃OD/D₂O (9:2 v/v) and (c) CH₃OH/H₂O (9:2 v/v) containing 100mM thymidine. All spectra recorded at 77K.
Figure 2.8 contd.. EPR spectra of a γ-irradiated frozen aqueous solution of methanol (9:2 v/v) containing 100mM thymidine, warmed to approximately (d) 115K and (e) 130K, revealing the thymine π*-anion. All spectra recorded at 77K.
After irradiation of the methanol/water system the frozen aqueous beads exhibit a violet colour. This is indicative of an electron trapped within the glass and the EPR spectrum shows the expected singlet at approximately 'free-spin' value, which is best observed in the deuterio-methanol/deuterium oxide system, figure 2.8b. At 77K the trapped electron \( e^- \) appears to be the only product of the electron-gain pathway since no hydrogen atoms or alkyl radicals are detected. However, it has been shown that \( 'CH_3' \) is also formed in this system and that these so called 'hot' methyl radicals react with nearby methanol molecules to give \( 'CH_2OH \) (17) (Symons and Eastland, 1977). It seems reasonable, therefore, to suggest that hydrogen atoms may proceed to react in the same way (16). In fact, Verma et al., (1969) demonstrated that photolysis of a \( \gamma^- \)-irradiated frozen aqueous solution of methanol induces a loss of \( e^- \) and a concurrent gain in the concentration of \( 'CH_2OH \) radicals.

A third radical is detected by EPR spectroscopy at 77K - although its concentration is relatively low. In \( CH_3OH/H_2O \) an anisotropic doublet is observed \( (A_{iso} \approx 130G) \), probably due to a radical species with coupling to just one \( \alpha \)-proton. On changing the solvent system to \( CD_3OD/D_2O \) the doublet disappears - presumably because the \( \alpha \)-proton has been replaced by an \( \alpha \)-deuteron. The radical is believed to be that of \( HCO \) (DCO).

### 2.3.4.2 The one-electron adduct of thymidine in methanolic glasses

Addition of a solute such as thymidine appears to yield little change upon the EPR spectrum at 77K, figure 2.8c. However, on closer inspection of the 'wings' of the spectrum the outer lines of the 5,6-dihydrothymine-5-yl radical ('TH) can be detected. By decanting liquid nitrogen the sample was allowed to warm and the spectrum carefully monitored until significant spectral changes were observed. The sample was then re-cooled and its spectrum recorded. The first changes seen upon warming are the reduction and ultimately the removal of both the trapped electron and the \( 'CH_2OH \) radical signals, figure 2.8d. This allows clear identification of 'TH and also reveals a well resolved doublet. The doublet arises from the thymine \( \pi^- \)-anion \( (A_{iso} \approx 12G, \) table 2.1) and persists right up to the melting temperature of the glass. 'TH, on the other hand, gradually decays upon further warming to leave only the \( \pi^- \)-anion, figure 2.8e. It should be noted that for reasons discussed earlier the protonation state of the \( \pi^- \)-anion cannot be determined.
Irradiation of the thymidine-containing deutero-methanol/deuterium oxide system produces a similar set of results. The electron, initially trapped at 77K, becomes mobile upon warming to form the thymine $\pi^*$-anion. The D-adduct radical 'TD is also observed both at 77K and after the initial anneal. However, as with 'TH, 'TD disappears on warming to higher temperatures leaving only the $\pi^*$-anion doublet.

At first glance these results seem somewhat at variance with those of Verma et al., (1969). In the current study no thymine $\pi^*$-anion doublet seems to be present at 77K - although it is possible that the signal may be superimposed upon that of the solvent triplet, i.e. 'CH$_2$OH. Furthermore, TH is found to decay rather than grow-in upon warming. The reasons for these differences are probably derived from the nature of the matrices used in each case. Verma et al. used methanol/water ratios of about 1:1 and added 0.5M NaOH to the system to assist the thymine base to dissolve.

**Scheme 2.3**

\[
\begin{align*}
T + e_{m^-} & \rightarrow T^- \quad (1) \\
T + H^- & \rightarrow TH \quad (2) \\
T^- + H^+ & \rightarrow TH \quad (3) \\
T^- + H^+ & \rightarrow T^-(H^+) \quad (4) \\
T^-(H^+) + H_2O & \rightarrow TH + H_2O \quad (5) \\
TH & \rightarrow \text{Diamagnetic products} \quad (6)
\end{align*}
\]

The mechanisms involved for this system are summarised in scheme 2.3. With or without solute, the net result of the electron-loss pathway from a methanol/water glass is the production of CH$_2$OH and that of the electron-gain pathway is a trapped electron. On warming, the trapped electron becomes mobile and either adds to the solute to form the thymine radical anion (T') (1), which may or may not protonate at a heteroatom (4), or reacts to give a hydrogen atom (11) and (13) [scheme 2.2]. The hydrogen atoms can then add across the C5-C6 double bond of nearby thymidine molecules to yield TH (2).
Protonation at the C6 position of the thymine radical anion (or \(\pi^*-\)anion) is perhaps expected to occur in a frozen aqueous methanol glass (3) and (5), in much the same way as it does for frozen aqueous chloride glasses. However, it would appear that this is not the mechanism of formation for \(\text{TH}^+\) in this case. Here \(\text{TH}/\text{TD}\) seems to form at 77K (probably by direct hydrogen atom addition across the C5-C6 double bond) and then subsequently decays on annealing to diamagnetic products (6).

### 2.3.4.3 Ethylene glycol (ethane-1,2-diol)

Good glass formation is found to occur at a much lower alcohol concentration for the ethylene glycol/water system. A previous study has shown that a suitable

**SCHEME 2.4**

\[
\begin{align*}
\text{H}_2\text{O} & \xrightarrow{\gamma} \text{H}_2\text{O}^+ + \epsilon_m^- & (1) \\
(CH_2\text{OH})_2 & \xrightarrow{\gamma} (CH_2\text{OH})_2^+ + \epsilon_m^- & (2) \\
\text{H}_2\text{O}^+ + \text{H}_2\text{O} & \rightarrow \text{H}_2\text{O} + \text{H}_2\text{O}^+ & (3) \\
\text{H}_2\text{O}^+ + \text{H}_2\text{O} & \rightarrow \text{H}_3\text{O}^+ + \text{OH} & (4) \\
(CH_2\text{OH})_2 + \text{H}_2\text{O}^+ & \rightarrow (CH_2\text{OH})_2^+ + \text{H}_2\text{O} & (5) \\
(CH_2\text{OH})_2^+ + \text{H}_2\text{O} & \rightarrow \text{CH(OH)}\text{CH}_2\text{OH} + \text{H}_3\text{O}^+ & (6) \\
(CH_2\text{OH})_2^+ + \text{ROH} & \rightarrow \text{CH(OH)}\text{CH}_2\text{OH} + \text{ROH}_2^+ & (7) \\
(CH_2\text{OH})_2^+ + \text{H}_2\text{O} & \rightarrow \text{CH}_2\text{OH}\text{CH}_2\text{O}^- + \text{H}_3\text{O}^+ & (8) \\
(CH_2\text{OH})_2^+ + \text{ROH} & \rightarrow \text{CH}_2\text{OH}\text{CH}_2\text{O}^- + \text{ROH}_2^+ & (9) \\
(CH_2\text{OH})_2 + \text{OH} & \rightarrow \text{CH(OH)}\text{CH}_2\text{OH} + \text{H}_2\text{O} & (10) \\
(CH_2\text{OH})_2 + \text{CH}_2\text{OHCH}_2\text{O}^- & \rightarrow \text{CH(OH)}\text{CH}_2\text{OH} + (CH_2\text{OH})_2 & (11) \\
\epsilon_m^- + \text{cavity} & \rightarrow \epsilon_l^- & (12) \\
\text{H}_3\text{O}^+ + \epsilon_m^- & \rightarrow \text{H}^- + \text{H}_2\text{O} & (13) \\
(CH_2\text{OH})_2 + \epsilon_m^- & \rightarrow \text{H}^- + \text{CH}_2\text{OHCH}_2\text{O}^- & (14)
\end{align*}
\]
(CH₂OH)₂ + H⁺ → CH(OH)CH₂OH + H₂  \hspace{1cm} (15)

ethylene glycol/water ratio is about 1:1 (Kaalhus and Johansen, 1973). The radiation chemistry of this system is essentially the same as that for methanol/water. Two major pathways are observed - electron-loss and electron gain. In the absence of solute both mechanisms lead ultimately to the formation of a carbon centred radical. These are summarised in scheme 2.4.

As for methanol no primary species (i.e. radical cations H₂O⁺ and (CH₂OH)₂⁺) are observed by EPR at 77K, figure 2.9a. Although probably formed, these react to give the more stable secondary product CH(OH)CH₂OH (Ershov and Pikaev, 1969; Lenherr and Ormerod, 1971). Its EPR spectrum consists of a triplet, figure 2.9b, which is derived from one α-proton coupling (A₁,ν ca. 20G) and one β-proton coupling (A₁,ν ca. 26G). There are two β-protons in this radical and both are θ-dependant. One β-proton is aligned with good σ-π overlap (i.e. θ = 0°) giving a large splitting, and the other with poor σ-π overlap (i.e., ~ 120°) giving an almost negligible splitting. A similar example can be see in chapter 3, figure 3.14. This radical is derived from (CH₂OH)₂⁺ and can be formed in two different ways: (i) Direct deprotonation of the radical cation at the carbon atom, (6) and (7), or (ii) deprotonation at the oxygen atom, (8) and (9), followed by hydrogen abstraction from a neighbouring alcohol molecule (11).

Violet beads are observed after irradiation of the ethylene glycol/water glass. This indicates the presence of trapped electrons. In the absence of solute these are removed on annealing and a concurrent increase in the CH(OH)CH₂OH radical observed (Ershov and Pikaev, 1969; Lenherr and Ormerod, 1971). Thus, trapped electrons, once mobile, can react to form hydrogen atoms (13) and (14), or possibly some form of alkyl radical, which can then in turn abstract a hydrogen atom from a neighbouring ethylene glycol molecule (15).

2.3.4.4 The one-electron adduct of TMP in an ethylene glycol/water glass

The EPR spectrum of a γ-irradiated ethylene glycol/water glass is somewhat different in the presence of TMP than in its absence, figure 2.9c. Superimposed upon
Figure 2.9. EPR spectra of γ-irradiated frozen aqueous solutions of (a) (CH$_2$OH)$_2$/H$_2$O (1:1 v/v) (b), (CH$_2$OH)$_2$/H$_2$O (1:1 v/v) warmed to approximately 140K and (c) (CH$_2$OH)$_2$/H$_2$O (1:1 v/v) containing 100mM thymidine. All spectra recorded at 77K.
Figure 2.9 contd.. EPR spectra of a γ-irradiated frozen aqueous solution of ethylene glycol (1:1 v/v) containing 100mM TMP, warmed to ca. (d) 130K, showing a growth of 'TH features, and (e) 150K, showing the growth of a quintet (marked e). All spectra recorded at 77K.
the solvent triplet and the trapped electron signal is a doublet ($\Delta H = 23G$). This has been identified by Kaalhus and Johansen (1973) upon subtraction of the solvent signals and assigned to the thymine $\pi^*$-anion. In addition to the doublet they found the 'TH radical at 77K. Both observations are confirmed in the present study.

On annealing to remove the trapped electrons an increase in both doublet and 'TH features is observed, concurrent with a decrease in 'CH(OH)CH$_2$OH, figure 2.9d. Features from a new radical now begin to appear and, on warming to the melting temperature of the glass, are revealed in the form of a quintet (marked $\epsilon$ on figure 2.9e). This quintet is virtually identical to those observed in the alkali-metal halide glasses. Kaalhus and Johansen (1973) suggested that it may be formed as a result of addition of 'CH(OH)CH$_2$OH across the C5-C6 double bond of thymine, which would give rise to species 2.7VII. If, as for OH-addition, the proton at C$_6$ is confined in such a way as to produce a hyperfine splitting of approximately 20G (and the methyl protons at C5 also give splittings of approximately 20G), then a quintet EPR spectrum could arise. This phenomenon of radical addition of species R (where R is a relatively 'bulky' species) to the C5 position of the thymine base may also explain the results obtained for $\gamma$-irradiated frozen aqueous TMP and will be discussed in chapter 5.

Many similarities exist between the methanol/water and the ethylene glycol/water systems. Both give rise to the formation of the thymine $\pi^*$-anion, although it is only observed on warming in the methanol case. Both give rise to 'TH at 77K - presumably by direct hydrogen atom addition. However, for ethylene glycol, on warming, the 'TH octet appears to grow-in initially and then decays to be replaced by a quintet. For methanol 'TH, formed at 77K, decays fairly rapidly leaving behind the thymine $\pi^*$-anion doublet.

2.3.5 The $\gamma$-irradiation of frozen aqueous solutions of sodium hydroxide

Many of the studies carried out on one-electron adducts to DNA bases have used sodium hydroxide glasses (Kaalhus and Johansen, 1973; Holdroyd and Glass, 1968; Srinivasan et al., 1969; Lion and van de Vorst, 1971) with a variety of alkaline concentrations ranging from 2M - 10M. The most commonly used concentration is 8M which yields an excellent glass and has therefore been used in the present study.

The radiation chemistry of frozen aqueous solutions of sodium hydroxide has two major pathways: (i) Electron-loss, leading to the formation of O$^-$ [(1)-(7) in
scheme 2.5], and (ii) electron-gain, forming either hydrogen atoms or electrons trapped within the glass [8 - (10), scheme 2.5]. With regards to the latter pathway, Moorthy and Weiss (1964), using concentrations of 2-3M, also demonstrated the presence of hydroxyl radicals as part of the electron-loss mechanism. However, these were not observed by either Blandamer et al. (1964) or Ayscough et al. (1965), using concentrations of 10M and 8M respectively, and can be taken as an indication of phase separation.

On γ-irradiation of an 8M NaOH/H2O glass at 77K, the resultant EPR spectrum consists mainly of a singlet at approximately the free-spin value, and a broad low-field feature, figure 2.10a. These features were previously noted in other studies (Blandamer et al., 1964; Moorthy and Weiss, 1964; Ayscough et al., 1965). The glassy beads are blue (λmax = 586nm) which, along with the EPR singlet, can be attributed to trapped electrons. Replacing NaOH/H2O with NaOD/D2O causes a considerable narrowing of the singlet. Blandamer et al. ascribed this phenomenon to an electron trapped at an anion vacancy. The broad resonance at g=2.0053 corresponds to the O' radical species.

Photobleaching the γ-irradiated samples of either NaOH/H2O or NaOD/D2O at 77K with (λ > 300nm) completely removes both the blue colour from the

SCHEME 2.5

\[
\begin{align*}
\text{H}_2\text{O} & \xrightarrow{\gamma} \text{H}_2\text{O}^+ + e_m^- & (1) \\
\text{OH} & \xrightarrow{\gamma} \text{OH} + e_m^- & (2) \\
\text{H}_2\text{O}^+ + \text{H}_2\text{O} & \xrightarrow{} \text{H}_2\text{O} + \text{H}_2\text{O}^+ & (3) \\
\text{H}_2\text{O}^+ + \text{H}_2\text{O} & \xrightarrow{} \text{H}_3\text{O}^+ + \text{OH} & (4) \\
\text{H}_2\text{O}^+ + \text{OH} & \xrightarrow{} \text{H}_3\text{O}^+ + \text{O}^- & (5) \\
\text{OH} + \text{OH} & \xrightarrow{} \text{O}^- + \text{H}_2\text{O} & (6) \\
\text{OH} + \text{H}_2\text{O} & \xrightarrow{} \text{O}^- + \text{H}_3\text{O}^+ & (7) \\
\text{e}_m^- & \xrightarrow{} \text{e}_i & (8)
\end{align*}
\]
Figure 2.10a. EPR spectrum of a γ-irradiated frozen aqueous solution of 8M sodium hydroxide, recorded at 77K, showing the trapped electron signal at approximately the 'free spin' value.
Figure 2.10b. EPR spectrum of O\(^{-}\), recorded at 77K, generated by photolysis ($\lambda > 300$nm) of a $\gamma$-irradiated frozen aqueous solution of 8M sodium hydroxide.
beads to reveal the $O^-$ spectrum, figure 2.10b. It can now be seen that the $O^-$
resonance is highly anisotropic ($A_\parallel = 2.0053$ and $A_\perp = 2.002$). It should also be noted
that a considerable loss of intensity for $O^-$ has occurred. Ayscough et al. (1965)
assigned this to a reaction between hydrogen atoms, formed by reactions (9) and (10),
and $O^-$ (12). The same phenomenon can also be seen on warming the glass - although
both $e_\pi^-$ and $O^-$ signals are lost over a wide temperature range (77-180K). Finally,
Blandamer et al. (1964) demonstrated that warming a photobleached sodium hydroxide
glass can regenerate the trapped electron signal. On the basis of this observation they
suggested that photobleaching the electron can induce occupation of a new trap site,
shallower than that giving rise to the blue colour and diamagnetic in nature. They
further suggested that the photoexcited electron can escape from its original cavity and
become trapped at another, which already contains an electron, to form an EPR 'silent'
pair ($e_\pi^-, e_\pi^-$).

2.3.6 The one-electron adducts of thymine derivatives in sodium
hydroxide glasses

2.3.6.1 Thymine and TMP

The presence of either thymine or TMP (100mM) in a sodium hydroxide glass
considerably reduces the trapped electron yield, and increases that of $O^-$ by
approximately 25%. This accords well with previous observations by Lenherr and
Ormerod (1971) and Kaalhus and Johansen (1973). Presumably this increase is due to
a competition for electrons between solute and $O^-$. For either the thymine or TMP
systems an EPR doublet is observed at 77K together with the familiar outer features of
$\text{TH}$, figure 2.11a. The doublet has been attributed previously to the thymine $\pi^*$-anion
(Aiso, ca. 12G, table 2.1). In fact, Kaalhus and Johansen (1973) have shown that its resolution is dependent upon the concentration of added solute and that this in turn corresponds to the presence of the trapped electrons. Thus, at high concentrations of solute little or no trapped electrons are present. The results using NaOD/D$_2$O are virtually identical. A $\pi^*$-anion doublet and lines corresponding to the C6 D-adduct radical (TD) are observed. Annealing or photobleaching these glasses, in the presence of thymine or TMP, yields an increase of $\mathbf{TH}$ - although this affect is small for thymine upon photobleaching (c.f. Lenherr and Ormerod, 1971). The increase of $\mathbf{TH}$ corresponds directly to a decrease of the $\pi^*$-anion doublet. This conversion is extremely efficient on annealing and was the basis on which Holroyd and Glass (1968) suggested that C6 protonation of the $\pi^*$-anion occurs. To investigate this suggestion in the present study both thymidine and its C6-deuterated analogue (2.III) were used as solutes in both NaOH/H$_2$O and NaOD/D$_2$O glasses.

2.3.6.2 Thymidine in 8M NaOH/H$_2$O

Results obtained for thymidine are identical to those for TMP. On photobleaching of either solute system an increase in $\mathbf{TH}$ octet is observed. Similarly, warming produces an increase in $\mathbf{TH}$ formation and decrease in $\pi^*$-anion doublet over the temperature range 77-190K, figure 2.11b and 2.11c. In the range 190-200K the glass begins to melt and $\mathbf{TH}$ decay sets in. It should be noted that residual hydrogen atoms, detected at 77K despite the presence of solute, disappear between 77-110K. Furthermore, O$^-$ is lost steadily over a much broader range (77-180K). By 190K only $\mathbf{TH}$ with a residual amount of $\pi^*$-anion doublet is detected, figure 2.11c. These results are in accord with previous observations of Holroyd and Glass (1968).

As explained earlier, the EPR spectrum of $\mathbf{TH}$ consists of eight lines spread approximately over 137G. The first two lines are separated by 20G. Pershan et al. (1964) first identified this splitting with the protons on the methyl group (table 1B.5). To account for the total width of the $\mathbf{TH}$ octet and the intensities of its lines Holroyd and Glass (1968) concluded that two $\beta$-protons at C6 must also be interacting with the unpaired spin, with an average splitting of 38.5G. However, they found that a poor experimental simulation was obtained if the two $\beta$-proton splittings are equal. A better fit can be obtained with splittings of 34G and 43G. When the solvent system was
Figure 2.11. EPR spectra of a γ-irradiated frozen aqueous solution of 8M sodium hydroxide containing 100mM TMP at (a) 77K, (b) 180K and (c) 190K, showing the concurrent growth of TH and decay of π*-anion doublet. All spectra recorded at 77K.
replaced by NaOD/D$_2$O eighteen lines spread over 112G were observed on annealing. This was interpreted in terms of the C6 D-addition radical (TD) formed by deuteronation (from the solvent) of the $\pi^*$-anion at the C6 position. A good simulation of this spectrum was obtained using a 20G methyl splitting, a 40G proton doublet and a 5.7G deuteron triplet splitting. Similar results have been found in the present study - see below.

2.3.6.3 Thymidine in 8M NaOD/D$_2$O

Figure 2.12 shows a series of spectra over the temperature range 77-200K for thymidine in NaOD/D$_2$O. As for the H$_2$O system the thymine $\pi^*$-anion doublet is observed at 77K. At higher temperatures, however, this is converted into TD - confirming the observations of Holroyd and Glass (1968). A relatively small but nonetheless detectable amount of TH is also observed to grow in as the temperature is increased. This is probably due to the presence of a small quantity of H$_2$O in the matrix (the preference for H-transfer corresponds to a kinetic isotope effect). On increasing the temperature TH is found to decay at a faster rate than TD. Thus, at 200K the 18 lines spectrum, observed previously by Holroyd and Glass, is now clearly resolved, figure 2.12d.

Holroyd and Glass (1968) noted that, by computer simulation of TH (as seen at the higher temperatures), a reasonable TH spectrum can only be obtained if an inequivalence of the two C6 $\beta$-protons is introduced (34G and 43G). Furthermore, they discovered that this inequivalence has to be reduced for a simulation of TD where one of the protons is replaced by a deuteron (5.7G, equivalent to a 37G proton splitting, and 40G respectively). These results are confirmed by simulation in the present study.

2.3.6.4 Thymidine-d6 (Thd-d6) in sodium hydroxide glasses

Replacing a proton with a deuteron at the C6 position of the thymine ring (2.III) causes a 'collapse' of the $\pi^*$-anion doublet ($\Delta H = 22.5$G for Thd in NaOH/H$_2$O) to a singlet ($\Delta H = 8.5$G for Thd-d6 in NaOH/D$_2$O) in either the H$_2$O or D$_2$O matrix, figure 2.13. This is clear proof that the thymine $\pi^*$-anion has a large spin density at the C6 position.
Figure 2.12. EPR spectra of a γ-irradiated frozen solution of 8M NaOD (in D₂O) containing 100mM thymidine at (a) 77K, (b) 180K and (c) 190K, showing growth of features assigned to the C6 D-adduct radical 'TD. All spectra recorded at 77K.
Figure 2.12d. EPR spectra of a γ-irradiated frozen solution of 8M NaOD (in D₂O) containing 100mM thymidine, warmed to 200K and recorded at 77K, showing the C6 D-adduct radical 'TD.'
Figure 2.13.

EPR spectrum of a γ-irradiated frozen aqueous solution of 8M sodium hydroxide containing thymidine-d6 (2.3), recorded at 77K, showing an unresolved singlet for the thymine π*-anion.
Annealing to higher temperatures gives rise to a conversion of both thymine \( \pi^- \)-anion singlets to multi-lined spectra. When thymidine-d6 in 8M NaOD/D\(_2\)O is warmed to 200K - to remove all residual TH - the spectrum shown in figure 2.14a is observed. By simulation it is possible to show that these fourteen lines correspond to the species TD(D), with both C6 protons exchanged for deuterons\(^3\). It can also be seen from simulation that, provided a sufficient linewidth is used, a reasonable 14 line spectrum is obtained from two equivalent C6 deuterons (average splitting of 6G, corresponding to a proton splitting of 39G) and a 20G methyl splitting, figure 2.14b.

For the NaOH/H\(_2\)O matrix the spectra are complicated by the presence of large amounts of TH. It is surprising that the concentration TH for this system is much larger than for the corresponding D\(_2\)O matrix. This is perhaps due to some form of substitution occurring at the base. Nevertheless, the spectrum of the species TD(H) is observed. It consists of a 20G methyl splitting, a 6.1G deuteron splitting (corresponding to a 40G proton splitting) and a 38G proton splitting [c.f. 34G for TH(H)]. Thus, it can be seen that an inequivalence of the C6 \( \beta \)-protons has to be introduced to obtain the correct intensities of the observed TH octet. Furthermore, this inequivalence is reduced when one of the \( \beta \)-protons is replaced by a deuteron and is lost altogether when both the \( \beta \)-protons are replaced by deuterons.

### 2.3.7 Frozen aqueous systems

Gamma-irradiated frozen aqueous solutions containing nucleotides or nucleotide complexes have been studied in great detail by Gregoli, Olast and Bertinchamps (1974, 1976, 1977a, 1977b, and 1979). One study in particular meticulously maps out an EPR temperature profile of \( \gamma \)-irradiated frozen aqueous TMP (termed dTMP by Gregoli et al., 1976). They suggest a dual pathway mechanism in such systems (i.e. electron-loss and electron-gain). Electron-loss is essentially indiscriminate, occurring at both base and sugar sites. Electron-gain, on the other hand, takes place only at the base to give a \( \pi^- \)-anion (c.f. aqueous glasses). On annealing, the \( \pi^- \)-anion doublet is converted, almost quantitatively, into an octet (TH). TH reaches a maximum value at ca. 225K.

\(^3\) To differentiate between the various 5-thymyl radicals the following nomenclature has been adopted: TH(H) corresponds to 'normal' TH; TD(D) to the species with two deuterium atoms at the C6 position (2.VI); TH(D) to 'normal' TD (2.IV); and TD(H) to Thd-d6 which has been protonated at C6 (2.VIII).
Figure 2.14. EPR spectrum (a) and simulation (b) of a γ-irradiated frozen solution of 8M NaOD (in D$_2$O) containing thymidine-d$_6$ (2.III), warmed to 200K and recorded at 77K, showing 14 lines assigned to the species TD(D), 2.VI.
Figure 2.15. EPR spectra of a γ-irradiated frozen aqueous solution of TMP (250 mM),
(a) warmed to 130 K and then (b) photolyzed (λ > 300 nm) for 20 minutes
at 77 K, showing a growth of TH features concurrent with a decay of
doublet assigned to the thymine π*-anion. All spectra recorded at 77 K.
and then decays at higher temperatures. At ca. 245K all electron-gain species are lost to reveal a quintet. The quintet, which is identical to those detected in the present study in alkali-metal chlorides and ethylene glycol glasses, was assigned to the C6 OH-adduct of thymine, 'TOH (2.V).

In the present study π*-anions are produced on annealing to 130K for both thymidine (ΔH = 24.6) and TMP (ΔH = 23.8) systems, figure 2.15a. On warming to this temperature, to remove hydroxyl radicals in the ice-phase (see chapter 5), 'TH is observed to grow-in. Further annealing of either TMP or thymidine systems increases the concentration of 'TH and decreases that of the π*-anion doublet. Similarly, photolysis for 20 minutes using an ultra-violet lamp, with a Pyrex filter (λ > 300nm), increases 'TH and decreases the intensity of the doublet, figure 2.15b. Thus, carbon atom protonation of the π*-anion can be induced both thermally and photolytically. The significance of these results, which are comparable to those for frozen aqueous DNA, will be discussed in detail in chapter 5.

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\(^4\) It should be noted that in all cases, unless otherwise stated, EPR spectra are recorded at 77K after warming to a particular temperature.
2.4 CONCLUSIONS

One-electron addition to thymine and its derivatives in non-acidic, frozen aqueous solutions of lithium chloride, sodium chloride, methanol and sodium hydroxide, at 77K, generates EPR spectra that consist mainly of doublets. The doublets, assigned to the thymine π*-anion, are derived from coupling to the anisotropic C6-H proton and have isotropic hyperfine coupling values of approximately 12G (table 2.1). That this doublet splitting arises from the proton at C6 is without doubt. Exchanging the proton with a deuteron causes the doublet to be replaced by a narrow singlet. No other hyperfine splittings are resolved within the linewidths of the doublets and thus the protonation state of the π*-anion in each case is unknown.

Protonation of the radical anion of thymine at a heteroatom site may take place but the present results, like those of previous authors, are inconclusive. The protonation state or the site of protonation is unknown. It is for this reason that the one-electron adduct of thymine is termed the π*-anion. However, the results for acidified lithium chloride glasses are interesting. As demonstrated by Bernhard and Patrzalek (1989) the π*-anion doublet is broadened significantly in the presence of HCl. This compares to uridine, where the uracil π*-anion doublet is not broadened in the presence of acid (chapter 4).

Protonation of the thymine π*-anion at a carbon atom site almost certainly takes place. As shown by Holroyd and Glass (1968), conversion of the π*-anion doublet into an octet occurs on warming in a γ-irradiated sodium hydroxide glass. That this protonation occurs exclusively at the C6 position is confirmed by the use of the C6-deuterated thymine analogue (2.III) in both NaOH and NaOD glasses. Simulation of the various 5-thymyl radicals, i.e. 'TH(H), 'TH(D), 'TD(H) and 'TD(D), can be achieved by having initially inequivalent proton splittings [i.e. 43G and 34G for 'TH(H)] which become increasingly equivalent when H is replaced by D.

The 5,6-dihydrothymine-5-yl radical, 'TH, is observed in many of the glasses at 77K. The C6 D-adduct, 'TD, is observed at 77K in the corresponding deuterium oxide systems. These radicals are most likely to be generated by direct hydrogen atom
addition at C6. It is interesting that no H-atom addition to C5 is observed, c.f. single crystal studies or studies where hydrogen atoms are produced from gaseous hydrogen (chapter 1B).

Finally, an EPR quintet is detected for some of the aqueous glasses (sodium chloride, ethylene glycol and possibly lithium chloride) and for frozen aqueous systems (TMP). It is only observed after annealing. Thus, it is not formed as a direct result of ionisation of the solvent medium at 77K. Gregoli et al. (1976) suggest that the quintet produced for γ-irradiated frozen aqueous TMP is derived from an OH-adduct to the thymine base (TOH). They claim that it is generated by hydroxylation of the thymine radical cation, 2.IX. However, they do not observe the radical cation at 77K, only its deprotonated successor, 2.X. This species, termed T4 by Gregoli et al., is unlikely to give rise to TOH. As will be discussed in chapter 5, T4 may add to the C6 position of the base of a neighbouring TMP molecule to give an EPR quintet in a similar way to 'CH(OH)CH2OH in the ethylene glycol glass.
Chapter Three

The site of protonation of one-electron-reduced cytosine and its derivatives in low-temperature matrices
3.1 INTRODUCTION

Electron addition to the nucleic acid base cytosine, its nucleosides and nucleotides gives rise to a $\pi^*$-anion (Dertinger, 1967; Herak and Galogaza, 1969; Lion and van de Vorst, 1971; Sevilla and van Paemel, 1972; Box et al., 1975; Westhof et al., 1975; Flossmann et al., 1976b; Herak et al., 1977; Close and Bernhard, 1979). The electron is accepted by the base into the lowest empty molecular orbital (LEMO) and resides primarily at C6, C4 and C2 of the pyrimidine ring (Baudet et al., 1962). The position of highest spin population, approximately 40%, is at C6, yielding a strong doublet in the EPR spectrum and having typically an isotropic 13-16G value (Table 1B.2).

Although the cytosine $\pi^*$-anion has been reported by many authors over the years, a great deal of uncertainty surrounds its true structure (i.e. its state and site of protonation). As with most thymine compounds, it is unclear as to whether any of the species reported in the literature are, in fact, pristine - i.e. unprotonated (1B.IV). However, it has been suggested that, in certain systems, protonation of various ring atom sites has occurred. Bernhard (1981) claims that EPR/ENDOR studies on single crystals of cytidine-3'-monophosphate (Box et al., 1975) and 2'-deoxycytidine-5'-monophosphate (Close and Bernhard, 1979) show spectra for the $\pi^*$-anion protonated at the N3 position. He suggests that this is due to the N3 site having a pK value of ca. 4 in the parent molecule (Ts'O, 1974), thus implying protonation of the parent molecule prior to electron addition. This proton coupling is considered too small to detect by EPR spectroscopy since the N3 proton is in a $\beta$-position to a site of significant electron spin density at C4, with a dihedral angle of 90° (3.1). Dertinger (1967), Westhof et al. (1975) and Herak et al. (1977) detect relatively small hyperfine coupling values for the C6-H proton and each group of workers concludes that protonation of the radical anion occurs at the exocyclic carbonyl oxygen O2 (3.II). In each case the O2-protonated species was only detected as a minority and could be obtained as a majority by irradiation of the single crystals at room temperature.
In studies using various low-temperature deuterated glasses, well resolved EPR doublets have been obtained. Sevilla and van Paemel (1972), using 8M NaOD/D$_2$O glasses, produced doublets which possess similar hyperfine coupling values to those observed in single crystal studies (Table 1B.2). These were assigned to the pristine radical anions. Sevilla and van Paemel also discovered that with cytosine a decrease in C6-H hyperfine splitting occurs upon addition of a substituent group to the N1 site.

Protonation at a heteroatom site of the pyrimidine ring, such as O2 or N3, is essentially reversible with little or no activation required to form the protonated species. However, as discussed in chapter 1B, protonation of pyrimidine radical anions is also possible at carbon atom sites and these reactions are effectively irreversible with a major activation barrier. Carbon atom protonation or hydrogen atom addition to cytosine is likely to occur across the C5-C6 double bond to give rise to the 5-yl (1B.V) and 6-yl (3.III) radicals. To distinguish between heteroatom and carbon atom protonation similar nomenclature to that used in chapter 2 has been adopted: C$^-$H$^+$ represents the radical anion protonated at either an oxygen or nitrogen atom, and CH the carbon atom protonated species (c.f. Bernhard, 1981).
3.2 EXPERIMENTAL

3.2.1 Materials

Cytosine (C), 1-methylcytosine (1mC), 3-methylcytosine (3mC),
5-methylcytosine (5mC), cytidine (Cyt), 2'-deoxyctydine (2'dC), cytidine-5'-monophosphate (5'CMP), 2'-deoxyctydine-5'-monophosphate (dCMP),
cytidylyl(3'→5')cytidine (CpC), cytidylyl(3'→5')guanosine (CpG), and N,N-dimethyl-2'-deoxyctydine (dm2'dC) were purchased from Sigma Chemical Company. Cytidine-d5,d6 (Cyt-d5,d6) was synthesized by Dr. A. Mather (Department of Chemistry, University of Leicester). Liquid helium was purchased from British Oxygen Company. All other materials were obtained as described in chapter 2.

3.2.2 Methods

The experimental procedure for work carried out in this chapter is outlined in chapter 2. Stock solutions of the various solvent systems were prepared and the beads formed in the usual manner.

Frozen aqueous samples were prepared by cooling, in liquid nitrogen, a Pyrex tube containing ca. 0.3ml of a given solution producing solid cylinders (pellets). For most substrates a concentration of 500 mM was used, but for cytosine and 3-methylcytosine only 35 mM and 60 mM respectively could be accommodated owing to their low solubility. The relevant EPR spectra for electron adducts in these systems were obtained by warming the pellets to 135K to remove masking 'OH radicals. Photolyses were carried out using an Oriel ultra-violet lamp directed through a Pyrex filter (λ > 300nm).

Samples were irradiated either at 77K using a Vickrad 60Co γ-ray source or at 4K with an X-ray source; irradiations were carried out with a dose of 0.5-1 Mrad for the aqueous glasses and 3 Mrads for the frozen aqueous systems. EPR measurements were made in the usual way.
NH
H O H X

OH
H
H
/
O —  P —  O'
/
o
O
CH,

Base (C or G)

3.VIII

3. IX

3. X

CYTOSINE

GUANINE

M+ O H

A-
### TABLE 3.1
Hyperfine splitting parameters for cytosine derivatives in various low-temperature matrices

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MATRIX&lt;sup&gt;b&lt;/sup&gt;</th>
<th>10M LiCl</th>
<th>5M LiCl + 3M HCl</th>
<th>5.5M NaCl</th>
<th>METHANOL</th>
<th>ETHYLENE GLYCOL</th>
<th>8M SODIUM HYDROXIDE</th>
<th>FROZEN AQUEOUS</th>
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<td>Cytosine (C)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
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<td>-</td>
<td>31.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39.5 - 32.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>29.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>26.3&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>D&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>25.4 (13.2)</td>
<td>-</td>
<td>24.4 (12.5)</td>
<td>25.3 (13.1)</td>
<td>-</td>
<td>23.8 (12.2)</td>
<td>25.2&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>1-methylcytosine (1mC)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>33.9 (24.0)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>25.7 (13.3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3-methylcytosine (3mC)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>38.7 (26.8)</td>
<td>-</td>
<td>36.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>39.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>38.5&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td></td>
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<td>26.7 (14.0)</td>
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<td>35.2 (25.1)</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
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<td>34.1 (24.2)</td>
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<td>30.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>39.5 - 28.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>36.2&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>25.5 (13.3)</td>
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<td>25.2 (12.9)</td>
<td>-</td>
<td>23.3 (12.0)</td>
<td>27.1&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>2'-deoxycytidine (2'dC)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>34.0 (24.1)</td>
<td>-</td>
<td>39.5 - 29.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>28.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>28.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
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<td>25.2 (12.9)</td>
<td>-</td>
<td>-</td>
<td>25.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Cytidine-5'-mono-phosphate (5'CMP)</td>
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<td>-</td>
<td>30.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>39.5 - 35.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>28.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>31.3&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
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<td>26.2 (13.7)</td>
<td>-</td>
<td>24.1 (12.3)</td>
<td>25.4 (13.2)</td>
<td>-</td>
<td>-</td>
<td>26.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> Two sets of values are used in this table. The main value is a measure of peak-to-peak distances of the outer lines of the triplets and doublets (i.e. ΔH); estimated error = ±0.3G. The values in parentheses are C6 isotropic hyperfine splittings as estimated by simulation of EPR spectra - see text.

<sup>b</sup> 1G = 10<sup>-4</sup>T.

<sup>c</sup> Only ΔH values are given.

<sup>d</sup> The values are expressed according to an annealing range; the first measurement was taken at approx. 130K and the second near to the melting temperature of the glass.

<sup>e</sup> ΔH value corresponds to a quartet - see text.

<sup>f</sup> Measurement taken close to the melting temperature of the glass - see text.
TABLE 3.1 (continued)
Hyperfine splitting parameters for cytosine derivatives in various low temperature matrices

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>H$_2$O/D$_2$O</th>
<th>10M LiCl</th>
<th>SM LiCl + 3M HCl</th>
<th>5.5M NaCl</th>
<th>METHANOL</th>
<th>ETHYLENE GLYCOL</th>
<th>8M SODIUM HYDROXIDE</th>
<th>FROZEN AQUEOUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2'-deoxycytidine-5'-monophosphate (dCMP)</td>
<td>H$_2$O</td>
<td>35.8 (25.6)</td>
<td>-</td>
<td>29.7$^c$</td>
<td>39.5 - 35.0$^d$</td>
<td>-</td>
<td>27.9$^c$</td>
<td>30.4$^c$</td>
</tr>
<tr>
<td></td>
<td>D$_2$O</td>
<td>26.5 (13.8)</td>
<td>-</td>
<td>23.8 (12.2)</td>
<td>25.2 (12.9)</td>
<td>-</td>
<td>23.0 (11.8)</td>
<td>25.5$^c$</td>
</tr>
<tr>
<td>Cytidylyl(3'→5')-cytidine (CpC)</td>
<td>H$_2$O</td>
<td>32.9$^c$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D$_2$O</td>
<td>25.0 (12.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytidylyl(3'→5')-guanosine(CpG)</td>
<td>H$_2$O</td>
<td>29.7$^c$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D$_2$O</td>
<td>25.0 (12.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N,N-dimethyl-2'-deoxyctydine</td>
<td>H$_2$O</td>
<td>25.5 (13.3)</td>
<td>-</td>
<td>24.4 (12.5)</td>
<td>25.3 (13.2)</td>
<td>-</td>
<td>24.1 (12.3)</td>
<td>-</td>
</tr>
<tr>
<td>(dm2'dC)</td>
<td>D$_2$O</td>
<td>25.3 (13.2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytidine-d5,d6 (Cyt-d5,d6)</td>
<td>H$_2$O</td>
<td>21.5$^c$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D$_2$O</td>
<td>8.5$^b$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytidine at 4K</td>
<td>H$_2$O</td>
<td>31.4$^c$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ $\Delta H$ corresponds to a poorly resolved doublet - see text.

$^b$ $\Delta H$ corresponds to an unresolved singlet - see text.
3.3 RESULTS AND DISCUSSION

3.3.1 The one-electron adducts of cytosine and its derivatives in lithium chloride glasses

3.3.1.1 Cytidine

The EPR spectra for the photo-oxidation of cytosine derivatives in aqueous LiCl/D_2O and NaOD/D_2O glasses containing potassium ferrocyanide [K_4Fe(CN)_6] exhibit anisotropic doublets (Sevilla and van Paemel, 1972; Sevilla et al., 1976). These have been described as representative of the pristine radical anion of cytosine with, for cytidine, an observed isotropic hyperfine splitting value of ca. 15G (Sevilla and van Paemel, 1972). As discussed in chapter 2, similar results were obtained for thymine derivatives; an EPR doublet was observed which derives mainly from the C6-H proton coupling. Liquid phase studies on thymine and uracil compounds have shown that this proton has an isotropic coupling value, equal to the average of the x, y and z components, of 11.8G. All other couplings are small and insignificant (Novais and Steenken, 1986). Unfortunately, no equivalent liquid phase EPR results have been obtained for cytosine or its derivatives.

In the present study a 10M LiCl/D_2O glass containing 100mM cytidine, prepared as described in chapter 2, yields a doublet virtually identical to that obtained by previous workers (Sevilla and van Paemel, 1972; Sevilla et al., 1976), figure 3.1. However, on changing the matrix to LiCl/H_2O the EPR spectrum of cytidine shows a well defined triplet, figure 3.2. The extra splitting, ca. 12G, that arises for cytidine in the H_2O glass is clearly due to an exchangeable proton derived from the solvent. This means that the species in these aqueous LiCl glasses is not the pristine radical anion (1B.IV) but a protonated form [C·'·(H+) and C·'·(D+)].

Protonation of the radical anion of cytosine (or π*-anion) at the C6 position of the pyrimidine ring should give rise to 'CH (1B.V). This can be excluded as a possible site of protonation here since it is an established reaction in pyrimidines, leading to an unmistakable sextet. A similar but less likely protonation at the C5 position (to give
Figure 3.1. EPR spectrum of a γ-irradiated frozen solution of 10M lithium chloride (in D₂O) containing cytidine (100mM), recorded at 77K, showing a doublet assigned to the species C⁻⁺(D⁺).
Figure 3.2. EPR spectrum of a $\gamma$-irradiated frozen solution of 10M lithium chloride (in H$_2$O) containing cytidine (100mM), recorded at 77K, showing a triplet assigned to the species C$^-$($H^+$).
3.III) should also produce a sextet. Both 1B.V and 3.III comprise one α- and two β-protons, the latter species giving smaller Δ, i.e. overall spectral width. Similar species are observed with uracil compounds - see chapter 4.

There are three possible sites for which protonation can be considered and which, in principle, might result in the EPR triplet spectrum: the ring nitrogen atom N3 (3.I), the exocyclic carbonyl oxygen atom O2 (3.II), and the exocyclic amino nitrogen atom N4 (3.IV). Involvement of the glycosidic nitrogen atom N1 can be excluded since replacement of cytidine with cytosine yields a nearly identical triplet pattern. The ring nitrogen atom at N3, which has been previously suggested as the favoured site of protonation (Bernhard, 1981), is not expected to produce a large proton hyperfine coupling since it possesses a low electron spin density. Indeed, for such a large proton splitting to be observed a considerable 14N splitting is also expected. For the carbonyl oxygen atom O2 protonation is likely to be in plane of the ring, and the hyperfine splitting, therefore, is also expected to be relatively small. Furthermore, it has been suggested that protonation of O2 causes a considerable reduction in the C6-H hyperfine splitting (Dertinger, 1967; Westhof et al., 1975; Herak et al., 1977).

Thus, ruling out 3.I and 3.II leaves only one real possibility; protonation at the amino nitrogen atom N4 (3.IV). Provided rotation of the resulting -NH3+ group is prevented by hydrogen bonds from nearby water molecules (3.V), one large coupling could result. This is despite the low spin density on the amino nitrogen atom itself. Indeed, it is possible to use the hyperfine coupling of the added proton to obtain an approximate value for spin-density at C4. The H2C-NH3+ radical is a simple model with almost unit spin density on carbon, and the three equivalent N-H protons give a hyperfine coupling of ca. 19G (Symons, 1973). This should be compared with the expected average for structure 3.IV, assuming free rotation of the -NH3+ group, of ca. 6G. Thus, a spin density on C4 of ca. 32% is obtained - very close to the literature value (Baudet et al., 1962). This large spin-density will enhance the basicity of the adjacent -NH2 group. It should be noted that the 14N coupling for the H2C-NH3+ radical is only 4G, and therefore the 14N coupling for structure 3.IV is expected to be small.

As discussed in chapter 2, a recent study on thymidine irradiated at 10K found a remarkably similar result (Sagstuen et al., 1989; Hole et al., 1991). They repeated an earlier experiment in which a single crystal of thymidine had been grown from D2O (Box and Budzinski, 1975). The deuterium oxide medium was replaced with water and
an extra doublet splitting of ca. 12G was detected. They suggested that protonation occurs at the carbonyl oxygen O4 in these crystals which, under normal circumstances, would not be expected to give a species with such a large proton hyperfine splitting - protonation being expected 'in-plane' of the pyrimidine ring. However, Young et al. (1969) have shown that the thymidine molecules are packed in the crystal in such a way that O4 is hydrogen bonded to an -OH proton that is held well out of the molecular plane (2.1). Addition of an electron to thymidine at low temperatures results in transfer of this proton within the hydrogen bond, giving strong overlap and a large hyperfine coupling due to hyperconjugation. In the LiCl/H2O glass no such packing exists and thus protonation at O4, if it occurs, would be expected 'in-plane' of the ring.

On warming the cytidine-containing LiCl/H2O glass an increase in the resolution of the triplet, and a slight increase in the triplet splitting $\Delta H$ are observed, figure 3.3. Upon further warming to the melting temperature of the glass, the triplet is retained without growth of other radicals. This is a little surprising since both thymidine and uridine in LiCl/H2O glasses give carbon atom protonated species on annealing (chapters 2 and 4 respectively). Furthermore, warming the deuterium oxide glass produces no significant alteration of the doublet, which remains right up to the melting temperature of the glass.

To determine the ease with which this N4-protonation of the cytosine radical anion takes place a cytidine-containing LiCl/H2O glass has been X-irradiated and observed by EPR at 4K. The resulting spectrum, although poorly resolved, clearly shows a triplet, figure 3.4. This indicates that protonation of the amino-group is still extensive even at this very low temperature and suggests that the -NH$_2$ 'lone-pair' is already hydrogen-bonded in the system prior to electron addition. Thus, proton-transfer is facile even at 4K.

### 3.3.1.2 Other cytosine derivatives

In order to provide more evidence for the postulate that the EPR triplet is indeed derived from the N4-protonated radical anion, a careful study of cytosine and modified cytosine derivatives in lithium chloride glasses has been carried out (table 3.1). The substrates examined include cytosine, 1-methylcytosine, 3-methylcytosine, 5-methylcytosine, 2'-deoxycytidine, cytidine-5'-monophosphate, 2'-deoxyctydine-5'-monophosphate.

$\Delta H$ is defined as the peak-to-peak splitting between the outer lines of the triplets ($Y_1$, figure 3.3a).
Figure 3.3. EPR spectra of a γ-irradiated frozen solution of 10M lithium chloride (in H$_2$O) containing cytidine (100mM) at (a) 77K and (b) 150K, showing increased resolution of the triplet. Both spectra recorded at 77K. For (a) $X_1 = \Sigma$ (the total splitting), and $Y_1 = \Delta H$ (the magnetic field separation of the peaks of the triplet outer lines).
Figure 3.4. EPR spectrum of a γ-irradiated (at 4K) frozen solution of 10M lithium chloride (in H$_2$O) containing cytidine (100mM), recorded at 4K, showing a poorly resolved triplet.
monophosphate, N,N-dimethyl-2'-deoxycytidine, cytidine-d5,d6, cytidylyl(3'→5')
cytidine and cytidylyl(3'→5')guanosine. These compounds can be broadly placed into
three categories: cytosine derivatives with modified substituents at the glycosidic
nitrogen atom N1 (C, 1mC, Cyt, 2'dC, 5CMP and dCMP), derivatives modified at the
base (3mC, 5mC, dm2'dC and Cyt-d5,d6), and dinucleoside phosphate derivatives
(CpC and CpG).

3.3.1.2.1 Cytosine derivatives with modified substituents at N1

Cytosine, 1-methylcytosine, cytidine, 2'-deoxycytidine, cytidine-5'-
monophosphate and 2'-deoxycytidine-5'-monophosphate all give good triplets in
LiCl/H2O and doublets in the corresponding D2O matrix. Inspection of the values in
table 3.1 reveals less than 1G total difference in ΔH for these triplets. Therefore, it is
suggested that all have readily formed N4-protonated radical anions.

3.3.1.2.2 Cytosine derivatives modified at the base

Cytosine derivatives with carefully positioned methyl groups have been used in
an attempt to 'block' potential sites of protonation. N,N-dimethyl-2'-deoxycytidine
(3.VI) possesses two methyl groups at the amino nitrogen N4. An EPR doublet and not
a triplet is obtained in the LiCl/H2O matrix, figure 3.5. Thus, addition of these methyl
groups causes the loss of the extra proton hyperfine splitting. There are three possible
explanations for this phenomenon: (i) The presence of the methyl groups has induced a
shift in the protonation site of the radical anion (possibly to N3), (ii) presence of the
methyl groups has prevented protonation of the radical anion altogether, and (iii)
protonation at N4 has still occurred but a subsequent reorientation of the -NH(CH3)2+2
+ group (due to a change in the hydrogen bonding environment) has reduced the coupling
to render it indistinguishable in the EPR spectrum. The first explanation seems to be the
most likely since a factor in amine protonation is that in order to become 'tetrahedral'
the two bulky methyl groups have to move considerably. Also, the two amino N-H
protons in the parent compound (2'dC) are acting as hydrogen-bond donors in the LiCl
solutions which will greatly enhance the basicity of the nitrogen lone-pair. This effect
cannot occur in the dimethyl derivative.

3-methylcytosine and 5-methylcytosine both give triplets in LiCl/H2O and
doublets in LiCl/D2O. The triplet splitting (ΔH) for 5-methylcytosine - a compound
Figure 3.5. EPR spectrum of a γ-irradiated frozen solution of 10M lithium chloride (in H₂O) containing N, N-dimethyl-2'-deoxycytidine (3.VI) recorded at 77K, showing a doublet (c.f. figure 3.2).
with a structure closely related to thymine - is very similar to that for the N1-substituted derivatives. 3-methylcytosine, on the other hand, gives about a 4G larger value (table 3.1). It is noticeable that the spectra for the latter, in both H₂O and D₂O, are considerably broader than for the N1-substituted derivatives. Nevertheless, the fact that with N3-methylation the triplet is retained strongly suggests that it cannot be a proton at this site that causes the extra 12G splitting. Indeed, it should be noted that of all the cytosine derivatives 3mC gives rise to the largest value for ΔH. This value increases even further on warming to ca. 39.5G.

Cytidine, deuterated at the 5 and 6 positions on the base (3.VII), gives rise to a poorly resolved doublet in the LiCl/H₂O matrix and a singlet in LiCl/D₂O, figures 3.6a and 3.6c. These EPR spectra, which can be simulated using a C₆ deuterium splitting of ca. 2G, figures 3.6b and 3.6d, also support the postulate that N4 is the site of protonation of the cytosine radical anion in lithium chloride glasses.

3.3.1.2.3 The dinucleoside phosphates CpC and CpG

Cytidylyl(3′→5′)cytidine and cytidylyl(3′→5′)guanosine (3.VIII) both give rise to triplet EPR spectra in LiCl/H₂O glasses. ΔH for CpG is considerably less, ca. 4G, than that for monomer cytidine (table 3.1). This reduction is reasonably explained in terms of a mixture of doublet and triplet features. The ΔH value for CpC is also less than that for monomer cytidine. Although small, ca. 1G, the reduction is nonetheless detectable. The significance of these observations and their relevance to polymeric and duplex systems, such as DNA, will be discussed later.

3.3.1.3 Protonation of the one-electron adducts of cytidine or dCMP in an acidified lithium chloride glass (5M LiCl + 3M HCl)

In a recent study by Barnes, Bernhard and Mercer (1991), using a cytidine-containing 8M HCl/H₂O glass, a quartet and not a triplet was observed. Furthermore, the corresponding deuterium oxide system (8M DCl/D₂O) yielded a broad but nonetheless clearly defined doublet. These observations are confirmed in the present study using acidified lithium chloride glasses (5M LiCl + 3M HCl/H₂O or 5M LiCl + 3M DCl/D₂O) containing either cytidine or dCMP. Indeed, warming both systems to remove solvent radicals produces a well resolved quartet (ΔH = 51G) and doublet (ΔH = 25.5G) respectively, figure 3.7. Barnes et al. went on to show that the quartet is
Figure 3.6. EPR spectra of γ-irradiated frozen solutions of 10M lithium chloride containing cytidine-d5,d6 (3.VII), (a) in H$_2$O, (b) simulation of (a), (c) in D$_2$O and (d) simulation of (c), showing singlets assigned to the cytosine π*-anion. All spectra recorded at 77K.
derived from the amino-protonated radical anion by using N,N-dimethyl-2'-deoxycytidine. This compound gives a triplet and not a quartet in 8M HCl/H$_2$O. In the light of present results it is somewhat surprising that a doublet is not obtained for dm2'dC. As suggested by Barnes et al., these results can only be reconciled by considering two exchangeable protons with significant splittings. If extensive protonation of the cytosine base is assumed in the acidified glass (i.e. to N3 and N4), prior to electron addition, a triplet could be expected for N,N-dimethyl-2'-deoxycytidine. However, to explain the observation of a quartet it is necessary to invoke a change in the hydrogen bonding environment around the -NH$_3^+$ group such that a 30° shift in angle θ occurs. Such a shift could now yield two, rather than one, extra proton hyperfine splittings, figure 3.14b.

3.3.2 The one-electron adducts of cytosine and its derivatives in sodium chloride glasses

As described in chapter 2, sodium chloride does not form a homogeneous glass, but upon freezing separates into two phases: an ice phase and a glass-like region. Irradiation of low concentrations of NaCl produces large amounts of OH and relatively small amounts of radicals associated with the glassy region (e.g. Cl$_2^-$). However, at high concentrations, such as 5.5M, this situation is reversed. Thus, 5.5M NaCl can perhaps be thought of as 'glass-like'. Cytosine, 3-methylcytosine, cytidine, cytidine-5'-monophosphate and 2'-deoxycytidine-5'-monophosphate, all give poorly resolved triplets in NaCl/H$_2$O (e.g. figure 3.8a) and doublets in the corresponding D$_2$O matrix (table 3.1). Like the aqueous lithium chloride glasses, 3-methylcytosine produces the largest ΔH (ca. 36G) and N,N-dimethyl-2'-deoxycytidine generates an EPR doublet (A$_{iso}$ ca. 13G). On warming, increased resolution of the triplets and also an increase in ΔH are observed, figures 3.8b.

3.3.3 The one-electron adducts of cytosine and its derivatives in alcoholic glasses

3.3.3.1 Methanol

As described in chapter 2, methanol/water at a ratio of 9:2 forms a neutral glass ideal for generating the one-electron adducts of pyrimidines. Thus, irradiation of a
Figure 3.7. EPR spectra of γ-irradiated frozen solutions of acidified lithium chloride containing 100mM dCMP, (a) in 5M LiCl + 3M HCl/H2O and (b) in 5M LiCl + 3M DCl/D2O.
Figure 3.8  EPR spectra of a γ-irradiated frozen solution of 5.5M sodium chloride (in H₂O) containing 100mM cytidine at (a) 77K and (b) 180K, showing increased resolution of the triplet. Both spectra recorded at 77K.
CD$_3$OD/D$_2$O glass containing 100mM 2'-deoxycytidine, and subsequent annealing to approximately 130K to remove solvent radical features, gives rise to an EPR doublet, figure 3.9b. In the corresponding CH$_3$OH/H$_2$O matrix a well resolved triplet is obtained, figure 3.9a. Like the lithium chloride glasses, these spectra can be assigned, respectively, to the N4-deuteronated and N4-protonated radical anions.

Upon further warming of the CH$_3$OH/H$_2$O glass, right up to its melting temperature, the triplet gradually disappears and is replaced by a broad doublet, figure 3.9c. There are three possible explanations for this phenomenon: (i) The deprotonation of C"(H$^+$), (ii) a net proton shift from the amino nitrogen atom N4, probably to the ring nitrogen atom N3, and (iii) rotational movement of the -NH$_3^+$ group. Deprotonation of C"(H$^+$) seems an unlikely event due to the facile nature of the protonation in the first place. The second and third explanations are both possible - the latter being favoured. On warming, the methanol/water matrix allows the -NH$_3^+$ group to rotate and, at temperatures around the melting point, the three protons are equivalent. Simulation shows that a broad doublet can be obtained from three equivalent (-NH$_3^+$) protons with $A_{iso} = 5.8G$, figure 3.9d.

For cytosine, cytidine, cytidine-5'-monophosphate and 2'-deoxycytidine-5'-monophosphate EPR triplets and doublets are also obtained in the respective H$_2$O and D$_2$O glasses. However, on further warming the cytosine and cytidine triplets collapse to broad doublets whereas those for 5CMP and dCMP remain essentially unchanged - right up to the melting temperature of the glass (table 3.1). This is somewhat surprising and is possibly related to the fact that both 5CMP and dCMP contain additional 5'-phosphate groups.

For N,N-dimethyl-2'-deoxycytidine it has been demonstrated that the two methyl groups act so as to 'block' the amino nitrogen atom as a potential site of protonation. Similarly, in CH$_3$OH/H$_2$O a doublet and not a triplet is observed ($A_H = 25.5G$) which, on further warming, remains unchanged.

3.3.2 Ethylene glycol (ethane-1,2-diol)

Ethylene glycol/water at a ratio of 1:1 forms a good glass. The EPR spectrum of an ethylene glycol/water glass containing 50mM cytidine, $\gamma$-irradiated and recorded at 77K, is shown in figure 3.10a. A very broad singlet feature is obtained, differing from that of either the solvent 'blank' or TMP spectra (chapter 2). On warming, this feature is converted into a triplet ($A_H = 42G$), figure 3.10b, which is believed to consist of
Figure 3.9. EPR spectra of γ-irradiated frozen aqueous solutions of
(a) CH$_3$OH/H$_2$O (9:2 v/v) containing 100mM cytidine, warmed to ca.
130K, (b) CD$_3$OD/D$_2$O (9:2 v/v) containing 100mM cytidine, warmed
to ca. 130K, (c) (a) warmed to near the melting temperature of the glass,
and (d) simulation of (c). All spectra recorded at 77K.
two independent triplets superimposed upon each other. The first is derived from the amino-protonated radical anion and the second from the ‘CH(OH)CH₂OH radical. On further warming, up to the melting temperature of the glass to remove all solvent features, the triplet is retained (ΔH = 36.2G), figure 4.10c, and is now almost certainly due solely to the N4-protonated cytosine radical anion. It is somewhat surprising that the triplet is not converted into either a species equivalent to that giving a quintet for TMP (i.e. structure 2.VI, chapter 2), or a broad doublet (c.f. 2'-deoxycytidine in CH₃OH/H₂O). It would appear, therefore, that the methyl group on the thymine base is largely responsible for the stability of structure 2.VI and thus its formation. It would also appear that the relative stability of the ethylene glycol glass over that of methanol is responsible for preventing rotation of the -NH₃⁺ group and thus no conversion to a doublet is observed.

3.3.4 The one-electron adducts of cytosine and its derivatives in alkaline (sodium hydroxide) glasses

Many of the studies carried out on DNA bases have involved the use of sodium hydroxide glasses to obtain π⁻-anions, and for cytosine and its derivatives this has mainly involved NaOD/D₂O glasses (Lion and van de Vorst, 1971; Sevilla and van Paemel, 1972). Although guanine, adenine and thymine give results that were easily interpreted, cytosine has caused some confusion due to the poorly resolved nature of its spectra.

In the present study, a broad unresolved singlet (ΔH = 28G) is obtained on irradiation of a 100mM cytidine-containing frozen solution of 8M NaOH/H₂O, figure 3.11a. On warming the glass through a series of temperatures (77-200K) slight variations in ΔH are revealed (both increase and decrease). At 150K there is an overall increase in ΔH of about 1.5G (to 29.5G) and, by 190K, ΔH has decreased to only 27G. Finally, at 200K - near to the melting temperature of the glass - the broad singlet resolves into a triplet (ΔH = 30G), figure 3.11b. When the solvent system is changed to 8M NaOD/D₂O the broad singlet is replaced by a narrower singlet (ΔH = 23G), which, on annealing to 130K, resolves into a doublet.

Other derivatives such as cytosine, 2'dC, 5CMP and dCMP give similar results. Broad, unresolved singlets in the H₂O matrix, which on warming to ca. 200K are resolved into triplets, and narrower singlets in NaOD/D₂O. Again, for N,N-dimethyl-2'-deoxycytidine and 3-methylcytosine in NaOH/H₂O a doublet (ΔH = 24G)
Figure 3.10. EPR spectra of a γ-irradiated frozen aqueous solution of ethylene glycol, (CH₂OH)₂/H₂O (1:1 v/v), containing 100mM cytidine at (a) 77K, (b) ca. 140K, (c) near to the melting temperature of the glass. All spectra recorded at 77K.
Figure 3.11. EPR spectra of a $\gamma$-irradiated frozen aqueous solution of 8M sodium hydroxide containing 100mM cytidine (a) at 77K and (b) warmed to 200K, showing increased resolution of the triplet. Both spectra recorded at 77K.
and a triplet ($\Delta H = 39G$) respectively are observed. This suggests that all other spectra observed for this system, i.e. for cytosine, cytidine, 2'dC, CMP and dCMP, contain doublet and triplet admixtures. The doublet contribution, however, is more significant than for other glasses and may derive from both unprotonated radical anion as well as the N3-protonated species.

3.3.5 Frozen aqueous systems

Gamma-irradiated frozen aqueous thymine compounds were briefly discussed in chapter 2 and will be discussed in more detail in chapter 5. Unlike aqueous lithium chloride, sodium hydroxide or alcoholic glasses, where the primary action is confined to electron addition, both electron-loss and electron-capture are expected for pure frozen aqueous solutions. Electron-gain for all cytosine compounds is believed to occur at the cytosine base and thus information about the protonation site of $C^-$ should be evident from the EPR spectra. Electron-loss, on the other hand, is less discriminate (from both the base and the sugar ) and the compounds can be classified, according to their radiation chemistry, into two groups: (i) Those which contain sugar groups attached to the N1 base site, and (ii) those which do not.

Cytidine, 2'dC, 5'CMP, dCMP and CpC are derivatives which contain a substituent sugar group. Electron-loss from a sugar moiety is generally favoured at -OH groups. For cytidine there are three such -OH groups, for 2'dC and CMP there are two, dCMP has one and CpC is a dimer containing four. Thus, the EPR spectra of these compounds in frozen aqueous media are complicated by mixtures of radicals. Nevertheless, when using H$_2$O a triplet is obtained, although somewhat narrower and less resolved than for aqueous lithium chloride and alcoholic glasses, figure 3.12a. Thus, protonation at the amino-nitrogen seems to have occurred to some extent, although the results suggest an EPR spectral mixture of doublet and triplet. Due to the broad and somewhat unresolved nature of the triplets, however, only $\Delta H$ splittings have been recorded in table 3.1. Mainly doublets are observed on replacing H$_2$O with D$_2$O, figure 3.12b, with a corresponding reduction in $\Delta H$ (table 3.1).

Electron-loss from either cytosine or 3-methylcytosine is obviously confined to the base itself. Despite the relatively low solubility of these compounds, it is still possible to obtain EPR spectra in both H$_2$O and D$_2$O. For cytosine the spectra are composite containing features for both base $\pi^*$-anions and $\pi$-cations, figure 3.12c. It is
Figure 3.12. EPR spectra of γ-irradiated frozen aqueous solutions of (a) CMP (0.5M) in H$_2$O (b) CMP (0.5M) in D$_2$O and (c) cytosine (35mM) in H$_2$O. All systems warmed to 130K and spectra recorded at 77K.
surprising, however, that a $\Delta H$ value of only 26G is observed (changing to 25G in $D_2O$) suggesting that little, if any, protonation of the radical anion occurs at the N4 site. Since N4-protonation seems to readily take place in frozen aqueous glasses this phenomenon is probably related to the fact that cytosine undergoes extensive base stacking in frozen aqueous solution (Ts'0, 1974). For 3-methylcytosine (in $H_2O$) the EPR spectrum is relatively 'weak' due to the low substrate concentration. Nevertheless, the results indicate that the radical anion appears to protonate strongly at the N4 site.

Gregori, Olast and Bertinchamps using frozen aqueous solutions of nucleotides produced a free-radical temperature profile. For dAMP (1974), TMP (1976) and dGMP (1977b) the radical anions (or $\pi^*$-anions) were found to convert, almost quantitatively, into H-adduct radicals (AH, TH and GH respectively). For dCMP (1979) the H-adduct radical 'CH was only detected when the system was exposed to light ($\lambda > 300nm$). In the present study the latter observation has been confirmed. On warming a $\gamma$-irradiated frozen aqueous solution of dCMP(0.5M) to 135K, a triplet-doublet EPR signal ($\Delta H = 30G$, table 3.1) is obtained, figure 3.13a. On further warming no features corresponding to 'CH are observed. However, if instead of further annealing the system is photolyzed for 20 minutes ($\lambda > 300nm$), a growth of features corresponding to a sextet are produced, figure 3.13b. These almost certainly derive from a carbon-atom protonated radical anion ('CH). This compares to the results for $\gamma$-irradiated frozen aqueous solutions of uracil compounds whereby a sextet, corresponding to the C6 H-adduct, is observed to grow-in both on warming and on photolysis. On further photolysis the spectrum remains unchanged. It is suggested, therefore, that carbon atom protonation of the cytosine $\pi^*$-anion can only be induced photolytically (c.f. TMP, chapter 2). On warming the photolyzed system, the 'CH sextet is then found to decay over the temperature range 77-230K. The relevance of these observations to frozen aqueous DNA is discussed in chapter 5.
Figure 3.13. EPR spectra of a γ-irradiated frozen aqueous solution of dCMP (0.5M in H₂O) (a) at 130K and then (b) photolysed (λ > 300nm) for 20 minutes. All spectra recorded at 77K.
3.4 CONCLUSIONS

One-electron addition to cytosine and its derivatives in non-acidic, frozen aqueous solutions of lithium chloride, sodium chloride, methanol, ethylene glycol, sodium hydroxide and water (or deuterium oxide) produces EPR spectra which contain either doublets, triplets or mixtures of both. The doublets, assigned to the deuteronated radical anion \([\text{C}'(\text{D}^+)\]) , are mainly derived from the usual pyrimidine anisotropic \(\text{C}_6-\text{H}\) proton coupling. The triplets, on the other hand, are characterised by an extra coupling to an exchangeable proton and are assigned to the species \(\text{C}^-\text{(H}^+)\). This extra coupling, ca. 12G, almost certainly derives from a proton at the amino-group nitrogen atom \(\text{N}_4\). Protonation at this site might have been expected to give a 1:3:3:1 quartet from three equivalent protons. However, if rotation is seriously restricted, then protonation can take place above (or below) the ring and may, in a protic glass, be 'fixed' by hydrogen-bonding. Thus, if the conformation is such that overlap is a maximum (figure 3.14a), i.e. \(\theta = 0\), and given a coupling of ca. 12G for this proton, then, according to the simple \(\cos^2\theta\) law (Symons, 1959), the other two protons should be approximately 3G. This splitting will be lost within the line-width of the spectra.

For LiCl/H\(_2\)O glasses, a variety of substrates have been examined and all, with the exception of N,N-dimethyl-2'-deoxycytidine, yield some form of triplet at 77K. These triplets range from a maximum splitting for 3-methylcytosine, to a minimum for CpG. For 3mC, the methyl acts as a 'blocking' group at the \(\text{N}_3\) site and thereby prevents its protonation. This leads to complete protonation at the amino-nitrogen atom. Similarly, N,N-dimethyl-2'-deoxycytidine contains two such 'blocking' groups which prevent protonation at the amino-nitrogen atom \(\text{N}_4\) and, therefore, presumably switch the protonation to the ring-nitrogen atom \(\text{N}_3\). For cytidine a \(\Delta\text{H}\) splitting of ca. 34G is obtained. On raising the temperature of the LiCl glass an increase in \(\Delta\text{H}\) of about 2G is observed. This can probably be attributed to relaxation of the hydrogen bonds around the \(-\text{NH}_3^+\) group. A similar, but more pronounced relaxation may have occurred for cytidine in the acidified lithium chloride glass. Protonation may occur at both \(\text{N}_3\) and \(\text{N}_4\) in the acidified glass and this is supported by the observation of a triplet for N,N-
Figure 3.14. Looking down the C4-N4 bond of the amino protonated cytosine radical anion shows the angle $\theta$ of a hydrogen atom relative to the p-orbital in (a) 10M LiCl and (b) 5M LiCl + 3M HCl. The hyperfine splitting, $A$, is proportional to $\cos^2\theta$, thus when $\theta \sim 0^\circ$ one large proton hyperfine splitting results. If $\theta \sim 30^\circ$ then two smaller, but significant, proton hyperfine splittings may result.
dimethyl-2'-deoxycytidine. For cytidine and dCMP, a 30° degree shift for $\theta$, figure 3.14b, gives rise to two additional proton hyperfine splittings of about the same value leading to a quartet.

Bernhard (1989a, 1989b) has shown that the EPR spectra of oligomers such as d(pCpGpCpG), irradiated in LiCl/H$_2$O glasses at 4K and observed using Q-band EPR spectroscopy, are doublets and not triplets. In the present study the dinucleoside phosphates CpG and CpC give a mixture of both doublet and triplet, with a larger doublet admixture than that which is seen for monomeric cytidine. Thus, a switch in protonation site of C' seems to occur on changing from monomeric to dimeric to duplex cytosine-containing systems. Although it is possible that this effect might be explained in terms of deprotonation of the radical anion, it seems very unlikely since it has been established that C' is a strong base, and is rapidly protonated in fluid aqueous solutions (Hissung and von Sonntag, 1979; Steenken, 1989). For G-C base pairs the central hydrogen bond, between the N1 proton of guanine and N3 of cytosine, is strong and short (3.1X). Thus, neutralisation of C' by the guanine N1 proton should be strongly favoured in cases where such pairs exist. The dinucleoside phosphate CpG probably forms a partial duplex system, giving a mixture of the species protonated at different sites. For the tetramer d(pCpGpCpG), however, a much better duplex is obviously obtained. CpC, however, does not form a duplex and yet a small reduction in $\Delta H$ is observed compared to monomeric cytidine. Indeed, results for polycytidylic acid (poly[C]) in frozen aqueous systems suggest an even larger reduction in $\Delta H$ (Malone, 1991). It is not clear why a strong doublet contribution is observed with polymeric cytosine derivatives.

In duplex systems, such as DNA, the cytosine amino-group acts as a proton-donor, whereas the N3 site acts as a hydrogen bond acceptor. The carbonyl oxygen O2 also acts as a hydrogen bond acceptor site and might be a possible alternative site for protonation of C'. However, this does not seem to be the case for monomeric cytosine derivatives since such a protonation would give rise to insignificant hyperfine coupling from the added proton (which is expected to remain in the plane of the pyrimidine ring), and the spin density on the oxygen is low. Also, in the present work, a doublet only is obtained for dm2'dC. This suggests a switch of protonation site from N4 presumably to N3. It is possible, however, that this switch of site might be to the carbonyl oxygen since the N3- and O2-protonated radical anions would give virtually identical doublets.
under our conditions. Careful single-crystal studies and/or ENDOR could solve this problem.

Although sodium chloride does not form a glass (c.f. lithium chloride) little, if any, electron-loss from the substrate seems to occur. However, triplets formed in NaCl/H2O are narrower than their LiCl/H2O counterparts (for CMP $\Delta H = 30$G and 35G respectively). On warming, a very significant increase in $\Delta H$ is observed - possibly due to relaxation of the hydrogen bonding network. It would therefore appear that extensive protonation at N4 occurs in sodium chloride glasses.

It has been suggested that the major reason for a preference of C$^{-}$ to protonate at N4 (in lithium chloride glasses) is a coordination of Li$^{2+}$ ions to the N3 site (Bernhard, 1990; Barnes et al., 1991; Hutterman et al., 1991). This would certainly prevent protonation at N3. However, the results obtained in alcoholic glasses where well resolved triplets are produced, suggest that this is not the case. Furthermore, a preponderence of direct bonding is not expected in the aqueous lithium chloride medium. Results obtained from infrared studies of frozen aqueous methanolic and electrolyte solutions show that solvent shared ion-pairs represent the major form of interaction (Strauss and Symons, 1977; 1979), and thus units such as 3.X are predicted. These should be good proton-donors.

The results obtained for aqueous methanolic glasses are particularly interesting. If protonation is 'locked' by hydrogen bonding and structure 3.V is correct then, on annealing, rotation or extensive libration might be expected to set in. This does not happen with glasses such as LiCl and NaCl, where perhaps only a relaxation of the hydrogen bonding network takes place. However, with methanol it would seem that rotation does eventually set in for cytosine derivatives such as cytidine and 2'dC. For compounds with a 5'-phosphate group, i.e. 5CMP and dCMP, the triplet EPR spectra remain 'fixed', $\Delta H$ ca. 35G, after an initial reduction of about 4G. The reason for this phenomenon is not entirely clear.

With sodium hydroxide, protonation of C$^{-}$ at the amino-group appears to take place to some extent. However, the doublet contribution of the EPR spectra obtained at 77K seems to be more significant than that obtained in other glasses. Due to the alkaline nature of the glass, it is possible that this doublet contribution is derived from unprotonated radical anion. However, a drawback to this is that a triplet is observed for 3mC with apparently no doublet admixture, which strongly suggests that protonation at N3 is involved in some way.
The relevance of these results in terms of polymeric and duplex systems such as DNA will be discussed in more detail in chapter 5. Clearly, monomeric cytosine derivatives cannot be used as 'benchmarks' for such systems, although other DNA base model compounds have proved in the past to be ideal. Finally, it is somewhat surprising that, considering the numerous studies carried out on cytosine and its derivatives, the triplet EPR spectrum had not been observed previously. It seems probable that many workers automatically used deuterium oxide as solvent in order to reduce linewidths.
Chapter Four

The generation of Uracil $\pi^*$-anions in low-temperature matrices
4.1 INTRODUCTION

Although not a DNA base uracil has been the subject of many free-radical studies. In particular, the pulse- or gamma-radiolyses of aqueous solutions and using either optical detection, conductivity measurements or in situ EPR spectroscopy (von Sonntag and Schuchmann, 1986; von Sonntag, 1987). These either involve monitoring reactions of OH or SO₄⁻, primarily associated with one-electron oxidation, with various uracil derivatives (Fujita and Steenken, 1981; Schuchmann et al., 1984; Schulte-Frohlinde and Hildenbrand, 1987; O'Neill and Davies, 1987; Schuchmann et al., 1987; Behrens et al., 1988; Fujita and Nagata, 1988; Fujita et al., 1988; Schulte-Frohlinde and Hildenbrand, 1989; Hildenbrand et al., 1989; Bothe et al., 1990; Hildenbrand, 1990), or monitoring reactions of H⁺ or aqueous electrons with the uracil derivatives (Das et al., 1985; Deeble et al., 1985; Novais and Steenken, 1986). Similar studies have also been carried out on aqueous solutions of polyuridylic acid [poly(U)] (Schulte-Frohlinde and Bothe, 1984; Deeble and von Sonntag, 1984; Lemaire et al., 1984; Schulte-Frohlinde et al., 1985; Deeble and von Sonntag, 1986).

In chapter 1B it was shown that one-electron reduction of (i.e. electron-addition to) uracil or uracil derivatives has been observed mainly with single crystals (Box et al., 1975; Flossmann et al., 1975b; Zehner et al., 1976; Bergene and Vaughan, 1976; Sagstuen (1980); Radons et al., 1981; Voit and Hüttermann, 1983), and low temperature aqueous sodium hydroxide glasses (Sevilla and van Paemel, 1972; van de Vorst, 1973). As with both thymine and cytosine one-electron addition to uracil gives rise to a π*-anion with a high electron spin density at the C6 position (approximately 50%). This of course yields the expected EPR spectral doublet having typically an isotropic 14G value. Sevilla and van Paemel (1972), using an 8M NaOD/D₂O glass, demonstrated such a doublet for uracil, uridine, 1,3-dimethyluracil and various 5-halouracils. They noted that, as in the cases for thymine and cytosine, uracil base gives a larger C6 isotropic hyperfine splitting than its derivatives substituted at the N1 position. Van de Vorst (1973), using an 8M NaOH/H₂O glass, further demonstrated the production of a doublet on addition of an electron to uracil, 2-thiouracil, 2'-
deoxyuridine and 2'-deoxyuridine-5'-monophosphate; although the spectra were somewhat poorly resolved. It should be noted that with both studies the exact nature of the species involved, i.e. its protonation state or site of protonation were unknown. The uracil $\pi^*$-anion has been observed and identified by EPR/ENDOR studies of single crystals of uridine-5'-monophosphate [Ba$^{2+}$ salt] (Box et al., 1975; Sagstuen, 1980; Radons et al., 1981), 1-methyluracil.HBr (Flossmann et al., 1975b), uracil-β-D-arabinofuranoside (Bergene and Vaughan, 1976) and 2'-deoxyuridine (Voit and Hüttermann, 1983). It is unclear as to whether or not any of these $\pi^*$-anions are pristine. However, Bernhard (1981) claims that for 1-methyluracil.HBr the uracil radical anion is almost certainly protonated and probably at the exocyclic carbonyl oxygen atom O4. He argues that this position may well be protonated in the parent molecule (Sobell and Tomita, 1964), HBr being the proton donor and thus capture of an electron would neutralize the positive charge. Also, he points out that from other single crystal studies on uridine-5'-monophosphate, uracil-β-D-arabinofuranoside and 2'-deoxyuridine C6 hyperfine splittings ranging from 12.8G to 14.1G are produced, smaller than that found for 1-methyluracil.HBr ($A_{12G}=14.7G$). He suggests, therefore, that the $\pi^*$-anion for 1-MeU.HBr has a larger C6 splitting due to its protonated state. Bernhard (1981) also claimed that a single crystal study on uracil, X-irradiated and observed at 300K, yields a uracil $\pi^*$-anion protonated at O4 (Zehner et al., 1976). However, as can be seen from table 1B.3, the C6 hyperfine splitting was found to be only 7G and Zehner et al. suggest that the radical is derived from H-atom addition to O4 with no mention of the $\pi^*$-anion.

In addition to studies using single crystals or low-temperature glasses one-electron adducts for uracil, 2-thiouracil, 1-methyluracil, uridine, uridine-5'-monophosphate and 2'-deoxyuridine-5'-monophosphate have been detected in aqueous solution (Novais and Steenken, 1986). Well-resolved EPR spectral doublets were observed allowing accurate isotropic hyperfine coupling constants to be assigned - see table 1B.3. As for thymine they found no splittings attributable to protons attached to either of the exocyclic carbonyl oxygen atoms. It should be stressed, however, that rapid protonation (relative to the EPR timescale) may still be occurring.

The protonation of pyrimidine radical anions at a heteroatom site (or sites) is a reversible reaction and has a small activation barrier. As has already been demonstrated for thymine derivatives, such a protonation in the solid state is extremely difficult to detect with EPR spectroscopy, whereas for cytosine the extra proton splitting can be easily detected. For uracil, heteroatom protonation might be expected to occur at either
of the two carbonyl oxygen atoms (O2 and O4) and, as for thymine, will be hard to
detect with EPR. However, protonation of the uracil radical anion at a carbon atom
position (an essentially irreversible reaction) is readily observed. Tables 1B.5, 1B.6
and 1B.7 show hydrogen-atom adducts at carbon positions for thymine, cytosine and
uracil derivatives. The C6 H-adduct for thymine (table 1B.5) is, of course, the 5,6-
dihydrothymine-5-yl radical, 'TH. It has been shown that protonation of the thymine
radical anion occurs exclusively at the C6 position. However, the position of carbon-
protonation of uracil and cytosine n*-anions is somewhat less certain. H-adducts at the
C6 position (1B.VIII) have been found in numerous single crystal studies for both
pyrimidines. For cytosine, the C6 H-adduct yields an EPR sextet, consisting of one α-
(Aiso ca. 17G) and two β-hyperfine splittings. The β-hyperfine splittings have been
found to vary slightly: β1 from 51-53G and β2 from 48-49G (Rustgi and Box, 1974;
Westhof et al., 1975; Flossmann et al., 1976a and 1976b). For uracil, a similar EPR
sextet is produced with one α-splitting of approximately 19G, a β1 splitting of 48-51G
and a β2 splitting of 38-45G (Flossmann et al., 1975a and 1975b; Bergene and
Vaughan, 1976; Sagstuen, 1980 and 1981; Radons et al., 1981; Voit and Hütttermann,
1983).

H-atom addition to C5 of uracil or cytosine (1B.VII and 3.III respectively) also
gives rise to an EPR sextet with one α- and two β-splittings. Although the α-splitting
is similar to those for cytosine and uracil C6 H-adducts, the β-splittings appear to be
significantly less; β1 is approximately equal to β2 and varying from 32-36G
(Flossmann et al., 1975b; Zehner et al., 1976; Flossmann et al., 1976b; Voit and
Hütttermann, 1983; Novais and Steenken, 1986). It should be noted however that
studies on 1-methylcytosine, uracil and uridine-5'-monophosphate (Rustgi and Box,
1974; Riederer et al., 1981; Radons et al., 1981) produced β-splittings which were
inequivalent, β1 varying from 45-49G and β2 from 20-30G. As with the C6 H-adducts
it is difficult to tell whether any of these species are formed by protonation of the n*-anion.
4.2 EXPERIMENTAL

4.2.1 Materials

Uracil (U), 1-methyluracil (1mU), uridine (Urd), 2'-deoxyuridine (2'dU), uridine-5'-monophosphate (5'UMP), 2'-deoxyuridine-5'-monophosphate (dUMP), uridylyl(3'→5')uridine (UpU) and ribonucleic acid were purchased from Sigma Chemical Company. All other materials were obtained as described in chapter 2.

4.2.2 Methods

The experimental procedure is as outlined in chapter 2. Stock solutions of the various solvent systems were prepared, and samples made up using concentrations of uracil derivatives of between 50-100mM. Frozen aqueous 5'UMP and uridine samples were made up to a concentration of 500mM. Beads were formed by allowing droplets of a given solution to fall into a reservoir of liquid nitrogen. Pellets were made by cooling a Pyrex tube containing 0.3ml of the said solution in liquid nitrogen. All samples were irradiated at 77K using a $^{60}$Co γ-ray source with a dose of 0.5-1.0 Mrad and EPR measurements taken in the usual way.

The EPR spectrum of the peroxyl radical (ROO$^\cdot$) was generated from a γ-irradiated frozen aqueous sample of RNA (50mg ml$^{-1}$) which had been exposed to oxygen gas for 10 minutes prior to freezing. The sample was then warmed to 130K, using the method described in chapter 2, to remove the hydroxyl radical signal from the spectrum. An unresolved singlet remains, figure 4.1, probably derived from a mixture of radical ions, i.e. pyrimidine radical anions (or $\pi^*$-anions) and purine radical cations (or $\pi$-cations). On further heating to approximately 230K the singlet is replaced by the highly anisotropic signal of the species ROO$^\cdot$, figure 4.2. It should be noted that the g-values of these radicals are virtually independent of the group R and that they are best observed at high microwave powers.
<table>
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<th>COMPOUND</th>
<th>$\text{H}_2\text{O}/\text{D}_2\text{O}$</th>
<th>10M LiCl</th>
<th>5M LiCl + 3M HCl</th>
<th>5.5M NaCl</th>
<th>METHANOL</th>
<th>ETHYLENE GLYCOL</th>
<th>5M SODIUM HYDROXIDE</th>
<th>FROZEN AQUEOUS</th>
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<td>-</td>
<td>-</td>
<td>24.9 (12.7)</td>
<td>23.8c</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>$\text{D}_2\text{O}$</td>
<td>24.9 (12.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>24.8c</td>
<td>23.7 (12.1)</td>
<td>24.7 (12.6)d</td>
<td>23.7 (12.1)</td>
<td>23.4 (12.0)</td>
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<td>24.5 (12.5)d</td>
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<td>-</td>
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<td>-</td>
<td>24.6c</td>
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* Two sets of values are used in this table. The main value is a measure of peak-to-peak distances of the outer lines of the doublets (i.e. $\Delta H$); estimated error $= \pm 0.3G$. The values in parentheses are $\Delta^i$ isotropic hyperfine splittings as estimated by simulation of EPR spectra - see text.

b $1G = 10^{-4} T$.

c Only $\Delta H$ values are given.

d Measurement taken close to the melting temperature of the glass - see text.

e $\Delta H$ value corresponds to a triplet - see text.
Figure 4.1. EPR spectrum of a γ-irradiated frozen aqueous solution of RNA
(50mg/ml), warmed to 130K, showing an unresolved singlet attributed to
electron-loss and electron capture centres at the purine and pyrimidine
bases respectively - c.f. DNA (figure 5.1).
Figure 4.2. EPR spectrum of a peroxyl radical (ROO•) generated as described in the text.
4.3 RESULTS AND DISCUSSION

4.3.1 The one-electron adducts of uracil and its derivatives in lithium chloride glasses

4.3.1.1

Holroyd and Glass (1968) first demonstrated the production of an EPR doublet ($A_{iso}$ ca. 16G, $\Delta H = 24G$) on addition of an electron to uracil in an 8M NaOH/H$_2$O glass. This observation was made after a photolyzed sample (\( \lambda > 254 \) nm) containing 0.5mM uracil and 5mM K$_4$Fe(CN)$_6$ was warmed to 190K. Subsequent studies on low-temperature aqueous glasses were carried out using either 8M NaOH/H$_2$O or 8M NaOD/D$_2$O. Sevilla and van Paemel (1972) detected well resolved EPR doublets for uracil, uridine, 1,3-dimethyluracil, 5-fluorouracil, 5-chlorouracil, 5-bromouracil and 5-iodouracil (tables 1B.3 and 1B.4). They noted the C6-H isotropic hyperfine splitting to be larger for uracil than for its derivatives and also showed that substitution at both N1 and N3 positions (1,3-dimethyluracil) reduces the hyperfine splitting even further. Van de Vorst (1973) detected doublets for uracil, 2-thiouracil, 2'-deoxyuridine and 2'-deoxyuridine-5'-monophosphate, although these were often somewhat poorly resolved.

The above studies appear to be unique for uracil derivatives in low-temperature aqueous glasses. There are no reports in the literature of studies involving low-temperature aqueous, aqueous alkali-metal chloride or aqueous alcoholic systems. Therefore, the aim of the present study was to carry out such experiments and hopefully extend our knowledge of one-electron reduced uracils. It should be interesting to compare these results to those both for single crystals and in the liquid phase.

A 10M LiCl/H$_2$O glass containing 100mM uridine, prepared as described in chapter 2, yields a doublet similar to those for thymidine (LiCl/H$_2$O and LiCl/D$_2$O) or cytidine (LiCl/D$_2$O), figure 4.3. The corresponding 10M LiCl/D$_2$O system containing...
Figure 4.3. EPR spectrum of a γ-irradiated frozen aqueous solution of 10M lithium chloride containing 10Mm uridine, showing a well-resolved doublet for the uracil $\gamma^\text{−}$-anion.
Figure 4.4. EPR spectrum of a γ-irradiated frozen solution of 10M lithium chloride (in D₂O) containing 100mM uridine, showing a well-resolved doublet for the uracil π*-anion.
100mM uridine also produces a well resolved doublet ($A_{iso} = 12.4 G$, $\Delta H = 24.3 G$), figure 4.4. The C6-H isotropic hyperfine splittings for these and other uracil derivatives in aqueous lithium chloride glasses were calculated by simulation of the experimental spectra. These splittings along with $\Delta H$ values are given in table 4.1. It is noticeable that both $\Delta H$ and C6-H hyperfine splittings are larger for uracil than for its derivatives. This agrees with the observations of Sevilla and van Paemel (1972) for all three pyrimidines, although the magnitude of the difference is somewhat smaller in the present study. It can also be seen that the C6-H isotropic hyperfine splittings for all uracil derivatives are larger than those for either thymine or cytosine derivatives in their respective glasses. The same, of course, cannot be said for $\Delta H$ values since a large extra 12G splitting is observed for many cytosine compounds in 10M LiCl/H2O - see chapter 3. The small, but noticeable, increased hyperfine splitting for uracil derivatives (i.e. 12.1 for uridine compared to 11.8 for thymidine or cytidine) is in agreement with molecular orbital calculations for these pyrimidines (Baudet et al., 1962).

The change of solvent from water to deuterium oxide usually produces a significant reduction in linewidths and consequently increases resolution of the EPR spectra. This is certainly the case for thymine and cytosine compounds (tables 2.1 and 3.1), although, for the latter, a triplet is converted to a doublet. Uracil is found to be no exception. On changing the solvent from H2O to D2O the lines are significantly sharpened and better resolution obtained, figures 4.3 and 4.4. Whether this line-sharpening is connected with a proton being exchanged at either of the carbonyl oxygen atoms [i.e. U"(H+) $\rightarrow$ U"(D+)] it is impossible to say. One interesting point arising from table 4.1 is that the $\Delta H$ values actually appear to increase slightly upon changing the solvent. It is not clear why this is the case.

On warming the 100mM uridine-containing 10M LiCl/H2O sample to 155K, using methods described in chapter 2, the solvent radical features are completely removed, figure 4.5a. These features for Cl^{-} and ClO^{-} are replaced by those of at least two other species. It can be seen from figure 4.5a that the uracil $\pi^*$-anion doublet remains upon warming to 155K. In addition two high-field lines, present at 77K, are now more clearly resolved. It is apparent that these lines, which increase in intensity upon warming to 155K, have two low-field counterparts (marked $\alpha$ on figure 4.5a). Indeed, the four lines are believed to be part of a sextet corresponding to the C6-H-adduct of uracil. Support for this postulate comes from both simulation, figure 4.6, and from the use of a LiCl/D2O glass. For the latter, the outer pairs of lines (high- and low-
Figure 4.5a. EPR spectrum of a γ-irradiated frozen aqueous solution of 10M lithium chloride containing 100mM uridine, warmed to 155K, showing the uracil $^{3}p$-anion doublet, features $\alpha$ (attributed to the O5H-adduct of uracil, 1B.VIII) and $\beta$ to the $\cdot$ of a peroxyl radical (ROO$'$).
Figure 4.5b. EPR spectrum of a γ-irradiated frozen aqueous solution of 10M lithium chloride containing 100mM uridine, warmed to near the melting point of the glass, showing features α, β, with the 1 for ROO' now present, and γ attributed to a quartet - c.f. figure 4.9.
Figure 4.6. EPR simulations of UH where (a) H-addition to C6 (1B.VIII) and (b) H-addition to C5 (1B.VII) of the uracil base occurs.
field) are replaced by a single pair of lines (better observed in the corresponding 5.5M NaCl/D2O 'glass', figure 4.8c) which strongly suggests that the species involved has had one of its protons replaced by a deuteron. Also present in the spectrum (figure 4.5a) is a feature marked β, better observed at high powers and can be interpreted as the parallel line for a peroxyl radical (ROO). The EPR spectrum of the peroxyl radical is virtually independent of the R group with \( g_i = 2.0033 \) and \( g_L = 2.0025 \), figure 4.2. ROO is generally formed by addition of molecular oxygen to a radical R'.

The effect of further warming on the uridine-containing lithium chloride glass can be seen with figure 4.5b. The outer lines of the sextet gradually disappear along with the uracil π*-anion doublet. The peroxyl radical signal, on the other hand, steadily grows-in. Thus it seems reasonable to suggest that a conversion of either the π*-anion or the C6 H-adduct (or possibly both) to ROO' has occurred.

Other uracil derivatives such as uracil, 1-methyluracil, 2'-deoxyuridine, uridine-5-monophosphate and 2'-deoxyuridine-5'-monophosphate exhibit similar EPR characteristics. In all cases the features, previously named as α and β, were observed; β, the parallel feature of the peroxyl radical, was found to vary in intensity according to the concentration of solute used. Features α, believed to be due to the C6 H-adduct of uracil, were replaced by a single pair of lines on changing the solvent to deuterium oxide.

4.3.1.2 The one-electron adduct of uridine or dUMP in an acidified lithium chloride glass (5M LiCl + 3M HCl)

A recent study by Patrzalek and Bernhard (1989) suggests that using an acidified lithium chloride glass can induce heteroatom protonation (presumably at the O4 position) of the thymine radical anion. The suggestion was made on the basis of an increase in the linewidths for the EPR doublet. In chapter 2 it was found that although the doublet is observed to broaden somewhat, both when compared to the spectra in the absence of HCl and to the deuterated system (i.e. 5M LiCl + 3M DCl/D2O), there is still no definitive proof of protonation. However, replacing thymidine or TMP with uridine or dUMP a much better resolved doublet is found in the acidified glass, figure 4.7. It is interesting that such a well resolved doublet is observed considering the fact that only the methyl group differentiates TMP and dUMP. Perhaps it is possible that the thymine radical anion is heteroatom protonated in a y-irradiated acidified lithium
Figure 4.7. EPR spectrum of a γ-irradiated frozen aqueous acidified solution of lithium chloride (5M LiCl + 3M HCl) containing 100mM dUMP, showing a doublet assigned to the uracil π*-anion.
chloride glass (possibly at O4) and that the uracil radical anion is not. However, it is difficult to see why this should be so.

4.3.2 The one-electron adducts of uracil derivatives in frozen aqueous solutions of sodium chloride

It was demonstrated, both in chapters 2 and 3, that a frozen aqueous solution of 5.5M sodium chloride provides an excellent system for observing pyrimidine electron adducts. Indeed, due to its stability on warming, the growth and decay of secondary species, such as TH, can be monitored more easily than with lithium chloride glasses. Using either uridine, 2'-deoxyuridine or uridine-5'-monophosphate (100mM) in a 5.5M NaCl/H2O 'glass' produces the now familiar EPR doublet, figure 4.8a. This, of course, is attributed to the uracil π* anion (protonation state unknown) and is also observed for the corresponding deuterium oxide glass. As seen previously for thymine and cytosine derivatives, the ΔH values for these doublets are notably smaller in sodium chloride than in lithium chloride (table 4.1). This presumably reflects the nature of the environment in which the π*-anion is formed (10M lithium chloride is a glass whereas a frozen aqueous solution of 5.5M sodium chloride is phase-separated to some degree).

Using 5.5M NaCl/H2O it is possible to obtain the one-electron adduct of RNA, figure 4.8b. Due to the lack of solubility of RNA in other aqueous glassy systems (such as 10M lithium chloride) its one-electron adduct can only be found in γ-irradiated frozen aqueous sodium chloride. The EPR spectrum at 77K of a γ-irradiated 50mg ml⁻¹ RNA-containing 5.5M NaCl/H2O glass consists of a broad doublet (ΔH = 25G). The doublet probably corresponds to one or both of the radical anions (or π*-anions) of the pyrimidines (i.e. cytosine and/or uracil). However, since no triplet (ΔH ~ 35G) is detected it seems reasonable to suggest that the amino-protonated species [C="(H⁺)] is not present.

On heating the NaCl/H2O 'glass' containing uridine (and similarly for 2'-deoxyuridine or 5UMP but not for RNA) solvent radical features gradually disappear to reveal two outer pairs of lines (c.f. LiCl). The high-field lines are easily observed at 77K and merely increase in intensity upon warming. The low-field lines, however, are disguised by the presence of solvent signals at 77K and are revealed only upon annealing to ca. 160K, figure 4.9a. These four lines, previously assigned as features α
Figure 4.8. EPR spectra of γ-irradiated frozen solutions of 5.5M sodium chloride:
(a) in H₂O containing 100mM 5'UMP (b) in H₂O containing 50mg ml⁻¹ RNA and (c) in D₂O containing 100mM 5'UMP. All spectra recorded at 77K.
Figure 4.9. EPR spectra of a γ-irradiated frozen aqueous solution of 5.5M NaCl containing 100mM 5'UMP warmed to (a) 160K, (b) 180K and (c) 200K, showing the growth and decay of features α to leave features γ, assigned as part of a quartet - c.f. figure 4.5b. All spectra recorded at 77K.
for the LiCl system, are believed to be part of a sextet and the other two lines are 'hidden' by the central doublet. Similarly, a low-field feature, previously assigned as \( \beta \), is believed to derive from the peroxyl radical \((\text{ROO}^\cdot)\). On further heating of the glass features \( \gamma \) are found to grow-in until, at temperatures near the melting point of the system, the EPR spectrum shown in figure 4.9c is observed. Similar features are observed using the lithium chloride glass, figure 4.5b.

The sextet is most probably derived from the \( \text{C}_6 \text{H} \)-adduct radical of uracil (1B.VIII). Table 1B.6 shows typical values for such a species. \( \Sigma \), i.e. the spectral width, is about 110G. If an isotropic \( \alpha \)-proton splitting of 20G is assumed then the two \( \beta \)-protons will be approx. 45G each. A simulation using these parameters is shown in figure 4.6a. It should be noted that the relative intensities of the lines for the sextet are 1:1:2:2:1:1. At 77K the outer pair of high-field lines are indeed at a ratio of 1:1. However, on warming to 163K, figure 4.9a, this ratio has changed considerably. An explanation for this can be found if some admixture of features \( \gamma \) is assumed for the latter. More evidence for the sextet being due to addition of a proton specifically at the \( \text{C}_6 \) position is seen by the use of the \( \text{D}_2\text{O} \) 'glass’. The outer pairs of lines are now replaced by a pair of single lines. This observation is supported by simulation on replacing a \( \text{C}_6 \) \( \beta \)-proton splitting of 45G by a \( \text{C}_6 \) \( \beta \)-deuteron splitting of 6.9G.

Simulation of the \( \text{C}_5 \) \( \text{H} \)-adduct, using parameters obtained either in aqueous solution at room temperature (Novais and Steenken, 1986) or low-temperature single crystal studies (Zehner \textit{et al.}, 1976), shows that \( \Sigma \) is only about 85-90G, figure 4.6b. This is derived from one \( \alpha \)-splitting of approximately 20G and two equivalent \( \beta \)-splittings of ca. 34G each. It should be noted that for several low-temperature single crystal studies on uracil derivatives the two \( \beta \)-splittings are found to be considerably inequivalent (table 1B.7). However, \( \Sigma \) remains about the same, i.e. 85-90G. Clearly, in the present case, the species observed to grow-in on warming belongs to the \( \text{H} \)-adduct with the larger value for \( \Sigma \). Thus, it is suggested that protonation of the radical anion (or \( \pi^* \)-anion) of uracil occurs at the \( \text{C}_6 \) position (c.f. \( \text{T}^+ \)) and not \( \text{C}_5 \).

\subsection*{4.3.3 The one-electron adducts of uracil derivatives in alcoholic glasses}

\subsubsection*{4.3.3.1 Methanol}

The \( \gamma \)-radiolysis of frozen aqueous solutions of alcohols containing pyrimidine bases, at specific alcohol/water ratios, gives rise to one-electron reduction of the bases
Figure 4.10. EPR spectrum of a γ-irradiated frozen aqueous solution of methanol (methanol/water 9:2 v/v) containing 100mM 2-deoxyuridine, warmed to approximately 130K and recorded at 77K, showing a well-resolved doublet assigned to the uracil π*-anion.
Figure 4.11. EPR spectrum of α-irradiated frozen aqueous solution of ethylene glycol (ethylene glycol/water 1:1, v/v) containing 10 mM 2-deoxyribose, warmed to near the melting point of the glass and recorded at 77 K, showing a doublet assigned to the unpaired electron.
(chapters 2 and 3). Thus, irradiation of a CH₃OH/H₂O (9:2 v/v) glass containing 100 mM 2'-deoxyuridine and subsequent annealing to approximately 130 K to remove solvent radical features yields an EPR doublet, figure 4.10. This doublet is well-resolved, has an isotropic hyperfine splitting of ca. 12.5 G and is retained up to the melting temperature of the glass without an observable successor. It corresponds to a uracil π*-anion and, as previously with thymine and cytosine compounds, is observed at the onset of melting. There are no signs of any carbon-protonated species previously detected for uracil derivatives in either lithium chloride or sodium chloride glasses. The results are almost identical when the corresponding CD₃OD/D₂O system is used. Upon warming to remove solvent radical features a well-resolved doublet only is obtained and it remains up to the melting temperature of the glass with no detectable successor. Similar results are also found with other uracil derivatives such as uridine and uridine-5'-monophosphate. The protonation state of thymine π*-anions generated in methanolic glasses is unknown (chapter 2). Similarly, for uracil derivatives the protonation state cannot be determined.

4.3.3.2 Ethylene glycol (ethane-1,2-diol)

The γ-irradiation at 77 K of an ethylene glycol/water glass containing 50 mM 2'-deoxyuridine and subsequent observation with EPR spectroscopy at 77 K reveals very broad, partially resolved features. Upon heating these are ultimately replaced by a doublet, figure 4.11, which is retained up to the melting point of the system. The doublet has a value for ΔH of ca. 24 G and can once again be clearly assigned to the uracil π*-anion.

For both irradiated methanol and ethylene glycol systems uracil π*-anions are observed. The mechanism of formation in each case is presumably the same as that suggested previously for thymine and cytosine compounds whereby electrons, initially trapped within the glass after irradiation at 77 K, become mobile upon annealing and add to the base moiety. It is unclear from the EPR spectra as to the protonation state of these uracil π*-anions.
4.3.4 The one-electron adducts of uracil and its derivatives in alkaline (sodium hydroxide) glasses

Studies by Holdroyd and Glass (1968), Sevilla and van Paemel (1972) and van de Vorst (1973) have demonstrated the effectiveness of a sodium hydroxide glass in producing one-electron adducts of uracil compounds (tables 1B.3 and 1B.4). In all cases EPR doublets, with a varying degree of resolution, were obtained. The study by the latter is of particular interest since it also involves the fate of these doublets upon heating the glass. Four derivatives were studied and only the spectrum for 2-thiouracil, observed upon warming to 160K, contained a sextet. The spectra recorded at 77K for uracil and dUMP consisted of the superimposition of 1:2:1 triplet upon a narrow singlet. For 2'-deoxyuridine a very complicated spectrum was observed both on warming and after photobleaching. Due to the somewhat ambiguous nature of these results it was decided to repeat this experiment and extend it further to other uracil derivatives.

Uracil, 1-methyluracil, uridine, 2'dU, 5'UMP, dUMP all give rise to similar EPR spectra when observed at 77K in a γ-irradiated 8M NaOH/H2O glass. A typical example is that of uridine, figure 4.12. An EPR doublet is obtained (table 4.1) together with a pair of lines which probably correspond to the C6 H-adduct. The doublets for all the uracil derivatives can be assigned to the uracil π*-anion with a typical ΔH value of ca. 23.5G.

Using the alkali-metal chloride systems (10M lithium chloride and 5.5M sodium chloride) a pair of lines is produced which can be assigned to the C6 H-adduct of uracil. In both cases the low-field pair of lines are obscured significantly by solvent radical signals. However, the use of the sodium hydroxide system, where the major solvent feature is O•-, allows the observation of the low-field lines at 77K, figure 4.12. It is also interesting to note the presence of weak features, marked δ on figure 4.12, inbetween each pair of lines. Although no positive identification of the species responsible for δ can be made the spectral width, Σ, appears to correspond to that of the C5 H-adduct radical of uracil, as shown by simulation (figure 4.6b). The four lines for the C6 H-adduct, marked α on figure 4.12, are detected by EPR for all the uracil compounds at 77K together with the π*-anion doublet. As discovered by van de Vorst (1973), the ultimate fate of these signals upon warming differs from compound to compound. For uracil, initial resolution of the central doublet precedes its decay and, ultimately, the formation of a complex composite spectrum, figure 4.13a. For uridine
Figure 4.12. EPR spectrum of a γ-irradiated frozen aqueous solution of 8M sodium hydroxide containing 100mM uridine, showing the uracil π*-anion doublet, features α (assigned to the uracil C6H-adduct) and features γ (assigned to the uracil CSH-adduct).
Figure 4.13. EPR spectra of γ-irradiated frozen aqueous solutions of 8M NaOH containing (a) uracil, (b) uridine, and (c) 2'deoxyuridine, all warmed to near to the melting temperature of the glass. All solutions were 100mM and spectra recorded at 77K.
Figure 4.13 cont. EPR spectra of γ-irradiated frozen aqueous solutions of 8M NaOH containing (d) 1-methyluracil and (e) uridine-5'-monophosphate, both warmed to near to the melting temperature of the glass. Both solutions were 100mM and spectra recorded at 77K.
and 2'dU initial warming produces a steady increase in intensity for the outer pairs of lines, i.e. the C6 H-adduct, followed by the formation of triplets, figures 4.13b and 4.13c. For 1-methyluracil, warming yields an increase in the C6 H-adduct species together with the δ features. On further annealing all features are replaced by yet another triplet, figure 4.13d, with a somewhat larger splitting than those observed for uridine and 2'dU. 5'UMP and dUMP follow a similar pattern of events. After the loss of initial radicals, i.e. the π*-anion doublet, the C6 H-adduct of uracil and the radical assigned to features δ, an unresolved doublet for 5'UMP (figure 4.13e) and an anisotropic triplet (relative line intensities of 1:1:1) for dUMP remain.

It is quite surprising that the spectra of the various uracil compounds in this matrix should differ so much near to the melting temperature of the glass. Even more so when comparing the spectra at 77K, i.e. all consist of the uracil π*-anion doublet, the C6 H-adduct of uracil and possibly the C5 H-adduct. Due to the variety of species observed at the higher temperatures and the fact that the only difference between the compounds is the pentose sugar moiety, it seems reasonable to suggest that these spectra may be derived from sugar radicals.

Finally, the studies by Holroyd and Glass (1968), Sevilla and van Paemel (1972) and van de Vorst (1973) produced poorly resolved EPR doublets. In the present study γ-irradiation of a sodium hydroxide glass containing any of the uracil derivatives generates well resolved doublets. These doublets correspond to the π*-anion of uracil (protonation state unknown).

4.3.5 Frozen aqueous systems

Gamma-irradiated low-temperature solutions of purine and pyrimidine compounds were shown by Gregoli et al. (1974, 1976 and 1977b), using EPR spectroscopy, to contain radicals derived from both electron addition to and electron abstraction from the base. However, in chapter 3 it was shown that the EPR signals for many cytosine compounds are dominated by a triplet (for H2O) and a doublet (for D2O) and that these features correspond to the cytosine π*-anion. Such domination of the spectra by the π*-anion suggests a preference for electron addition to, rather than electron abstraction from, the base. A similar tendency is observed for frozen aqueous uracil compounds such as uridine, 2'dU, UpU and dUMP. EPR doublets, completely dominating the spectra in each case, indicate the presence of the uracil π*-anion, figure 4.14b (table 4.1). This is somewhat surprising in view of the results obtained by
Figure 4.14. EPR spectra of γ-irradiated frozen aqueous solutions of (a) 5'UMP (500mM) and (b) uridine (500mM), warmed to 130K and recorded at 77K.
Gregoli et al. for gamma-irradiated frozen aqueous dAMP (1974), TMP (1976) and dGMP (1977b) and possible reasons for such a phenomenon will be discussed in chapter 5.

Gamma-irradiated frozen aqueous uracil and 5'UMP exhibit different EPR characteristics from those of other uracil derivatives. The spectrum for uracil is remarkably similar to that for cytosine (in D$_2$O) and appears to be a mixture of electron-loss and electron-gain features. 5'UMP, on the other hand, seems to contain no uracil $\pi^*$-anion doublet at all, figure 4.14a. Even accounting for the fact that the spectrum is a composite $\Delta H$ is now only 19G (in either H$_2$O or D$_2$O).

On heating, stepwise, the various uracil systems a series of complex EPR spectra are obtained. In many cases no formation of the uracil base C6 H-adduct is observed. However, for dUMP an initial growth of the pairs of lines, previously assigned to the H-adduct, is found. In chapters 2 and 3 radicals assigned to a H-adduct were induced by photolysis ($\lambda>300$nm), following the procedure of Gregoli et al. (1974, 1976, 1977b). Similarly, for dUMP it is found that after warming to 130K to remove the ice phase OH radicals, the EPR doublet (from the uracil $\pi^*$-anion) diminishes on stepwise photolysis with a concomitant growth of a pair of lines. These lines, identical with those observed for sodium chloride, lithium chloride and sodium hydroxide suggest the formation of the C6 H-adduct radical of uracil. Although only four lines are resolved it seems reasonable to suggest that once again photolytically-induced carbon atom protonation of the $\pi^*$-anion has occurred.
4.4 CONCLUSIONS

Addition of an electron to uracil and its derivatives in low-temperature aqueous, aqueous lithium chloride, aqueous acidified lithium chloride, aqueous sodium chloride, aqueous alcoholic (methanol and ethylene glycol) and aqueous sodium hydroxide systems gives rise to a doublet (\(A_{\text{iso}}\) ca. 14G), as observed by EPR spectroscopy. The doublet is derived from coupling to the proton at C6 of the uracil ring and is undoubtedly due to the uracil radical anion (or \(\pi^*\)-anion). In all cases the protonation state of this radical anion is unknown. As explained in previous chapters, a proton added to either carbonyl oxygen atom would be expected to lie in the plane of the ring and the hyperfine coupling therefore is expected to be relatively small. Thus, any hyperfine structure which may be due to such a proton is unresolved.

For aqueous lithium chloride, aqueous sodium chloride, aqueous acidified lithium chloride and aqueous sodium hydroxide matrices H-adducts of uracil are detected at 77K. Except for sodium hydroxide only the C6 H-adduct (1B.VIII) is observed. Since a net reduction in signal intensity for H is also observed at 77K, relative to the spectrum in the absence of solute, it is suggested that direct hydrogen atom addition (at the C6 position) has occurred, thereby yielding \(\text{UH}\). Proof of the presence of this radical is obtained by simulation, figure 4.6. With the exception of the alcoholic glasses, warming these systems results in the gradual replacement of the uracil \(\pi^*\)-anion doublet by the uracil C6 H-adduct signal. Thus, as with thymine derivatives, carbon atom protonation of the uracil radical anion (or \(\pi^*\)-anion) occurs exclusively at the C6 position. This is confirmed by the use of deuterium oxide glasses whereby a 45G \(\beta\)-proton splitting is replaced by a 7G \(\beta\)-deuteron splitting.

An interesting point arises from the fact that both thymine and uracil \(\pi^*\)-anions undergo carbon atom protonation but that cytosine \(\pi^*\)-anions do not. It has been demonstrated conclusively that the cytosine radical anion undergoes heteroatom protonation in many low-temperature aqueous glasses (almost certainly at the exocyclic amino nitrogen atom N4, chapter 3). However, the protonation states of uracil and thymine are unknown. Thus, it is possible that these radical anions are pristine at 77K.
and consequently give rise to carbon atom protonation on warming. On the other hand, the cytosine radical anion, which \textit{is} heteroatom protonated at 77K, seems unable to protonate at a carbon atom site.

The results for sodium hydroxide glasses containing various uracil compounds are somewhat in contrast with those of other 'glassy' systems. At temperatures near to the melting point of the glass, the spectra for uracil compounds in both lithium chloride and sodium chloride contain features $\beta$ and $\gamma$, the former assigned to a peroxyl radical ROO$. At temperatures near to the melting point of the sodium hydroxide glass a variety of spectra are observed. These spectra may be due to different sugar radical species.

Addition of an electron to ribonucleic acid in low-temperature frozen aqueous 5.5M sodium chloride also gives rise to an EPR doublet. Although the doublet is poorly resolved there can be little doubt that electron addition has occurred to one or both of the pyrimidine bases, since the spectra for the radical anions (or $\pi^*$-anions) of the purines consist of narrow singlets (Bernhard, 1989b). If electron addition has taken place at cytosine then protonation of the amino group must somehow be prevented, since no triplet is generated ($\Delta H \sim 35G$). This compares to results in chapter 3 for polymeric cytosine containing systems.
Chapter Five

The effects of ionising radiation on frozen aqueous DNA
5.1 INTRODUCTION

As described briefly in chapter 1A, it is now well established that γ-irradiation of DNA, both in vivo and in vitro yields single- and double-strand breaks (SSBs and DSBs respectively), along with modification and release of bases (Hüttermann et al., 1978). The damage caused by such events can be repaired in the cell with varying degrees of efficiency (Lehmann and Bridges, 1977). Although it is uncertain as to which kind of damage is cytotoxic or mutagenic it is generally agreed that strand break formation is serious, interrupting the function of DNA.

To probe the mechanism of radiation-induced strand break formation it is necessary to use in vitro experiments which relate to DNA in the cell. As discussed in chapter 1A, the effects of radiation upon a medium can be divided into two classes - the so-called 'direct' and 'indirect'. These are two limiting cases for the effects of radiation upon a particular system. The 'direct' effect has been defined in terms of damage produced in the same molecule in which the primary processes take place. The 'indirect' effect has been defined as damage produced by diffusible radical products of the suspension medium of the molecule of interest, usually water (Dertinger and Jung, 1969). In reality, damage to many of the γ-irradiated DNA systems studied in vivo probably lies somewhere in between these two extremes.

Although water can account for approximately 75% of the weight of most cells, much of it may well be taken up as a hydration shell (Tao et al., 1989). Due to the hydrogen bonding environment of this solvation water, radiation damage (i.e. electrons or positive 'holes') is more likely to be transferred directly to DNA than be trapped within the water molecules (to form diffusible radicals which can then attack DNA). Thus, although the 'indirect' damage route almost certainly dominates for the γ-irradiation of fluid aqueous solutions, as will be shown later it is unlikely to play a significant part for the γ-irradiation of 'dry' systems such as frozen aqueous DNA.
5.1.1 Studies of direct damage to DNA

The direct damage mechanism results in the formation of electron-gain and electron-loss centres upon the DNA itself. On γ-irradiation of dilute aqueous solutions of DNA the probability of forming such centres is low (Inokuti, 1983). It might be expected that solvated electrons, formed by the radiolysis of water, would react with the DNA to form radical anions of one or more of the bases. It is interesting to note, however, that these electrons, which react readily with the pyrimidine bases, nucleosides and nucleotides (at nearly diffusion control rates), react relatively slowly with polynucleotides such as poly[U] and, in particular, DNA (for reviews see von Sonntag and Schuchmann, 1986; von Sonntag, 1987).

It is possible to induce electron-loss from polynucleotides in dilute aqueous solutions by the use of a laser (Schulte-Frohlinde et al., 1985). In anoxic dilute aqueous solution of poly[U] photoionisation by laser light, a biphotonic process, gives rise to electron-loss at the uracil base which is then followed by SSB formation. Schulte-Frohlinde et al. deduced that direct damage on the uracil base leads ultimately to the same chemical pathway for SSB formation as that induced by hydroxyl radicals.

Direct damage is usually observed to occur in systems in the solid state, where the amount of water is relatively low and usually taken up in solvation; i.e. 'dry' DNA (including oriented fibres), frozen aqueous DNA, single crystals of bases, nucleosides and nucleotides, and low-temperature aqueous glasses of DNA constituents. The main technique for monitoring such damage is EPR. The effects of ionising radiation upon low-temperature aqueous glasses containing pyrimidine derivatives has been discussed in great detail in chapters 1B, 2, 3 and 4 along with single crystal studies of pyrimidine bases, nucleosides and nucleotides.

Ionisation of 'dry' or frozen aqueous DNA is essentially indiscriminate, i.e. electron-loss occurs at the solvating water, the sugar-phosphate backbone and the bases. However, it has been demonstrated by EPR that the only surviving radical species after irradiation at 77K are on the DNA bases (Graslund et al., 1971; Graslund et al., 1975; Sevilla, 1977; Gregoli et al., 1982). Thus, movement of radical centres must take place. The partial overlapping of the π-orbitals in the stacked bases has been suggested as the medium most likely for migration of electrons and holes along the duplex until their ultimate trapping at specific sites (Gregoli et al., 1977b; Gregoli et al., 1979; Sevilla, 1977).
5.1.2 Frozen aqueous systems

Unlike the ionization of 'bulk' water at room temperature, free radicals produced as a result of γ-irradiation of ice are trapped in the matrix and can easily be monitored by EPR spectroscopy at low temperatures. The ejection of an electron, giving rise to the radical cation of water, is considered as the major primary event, (1) in scheme 5.1.

SCHEME 5.1

\[
\begin{align*}
H_2O & \xrightarrow{\gamma} H_2O^+ + e^- \\
H_2O^+ + e^- & \rightarrow [H_2O^+] \xrightarrow{} H^+ + OH^- \\
H_2O^+ + H_2O & \rightarrow H_2O + H_2O^+ \\
H_2O^+ + H_2O & \rightarrow OH^- + H_3O^+ \\
H_3O^+ + e^- & \rightarrow H^+ + H_2O \\
H^+ + H_2O & \rightarrow H_2 + 'OH '.
\end{align*}
\]

Neither H_2O^+ nor trapped electrons (e^-) are observed by EPR spectroscopy at 77K or below. Electron return will yield H_2O molecules in excited states which can then decompose to H^+ and 'OH radicals (2). The excited H_2O molecules may also fall into the ground-state and thus no net reaction has occurred. This will simply reduce the measured quantum yield or G-value.

The radical cation of water (H_2O^+) may undergo hole transfer with a neighbouring water molecule, until being trapped as 'OH, i.e. (3) and (4) in scheme 5.1. This is expected to be extremely rapid since the proton involved is already hydrogen-bonded to the acceptor water molecule. The mobile electron (e^-) can react
with a proton (i.e. $\text{H}_3\text{O}^+$) to form $\text{H}^-$, with an increased G-value (for review see Symons, 1982a).

EPR spectroscopy, in fact, detects only 'OH in $\gamma$-irradiated ice at 77K. The $\text{H}^-$ signal, which can be assigned quite unambiguously, is detected at 4K but on annealing to about 20K decays via a complicated kinetic pathway (Fluornoy et al., 1962). Probably the main reaction of its decay is (6) in scheme 5.1. The 'OH signal when first seen was assigned as $\text{H}^-$ (with a large decrease in hyperfine splitting from the gas phase value of 508G) or as $\text{HO}_2^-$ radicals (Matheson and Smaller, 1955; Siegel et al., 1960), and later as $\text{H}_2\text{O}^+$ ions (McMillan et al., 1960). It consists of a 40G doublet (Brivati et al., 1965) and its spectrum changes on cooling to 4K (Symons, 1970; Johnson and Moulton, 1978). This has been shown to be due to deprotonation of 'OH, giving rise to $\text{O}^-$ (Symons, 1982b).

### 5.1.2.1 DNA model compounds

The above processes have been shown to occur in frozen water in the absence of solutes. The presence of a solute considerably changes the nature of the system. If that solute is a nucleotide, i.e. 2'-deoxyadenosine-5'-monophosphate (dAMP), then on freezing its molecules are found to be excluded by the ice and coerced into a separate phase (Gregoli et al., 1974).

Initial studies upon $\gamma$-irradiated frozen aqueous solutions of nucleotides and nucleotide-complexes were carried out by Gregoli, Olast and Bertinchamps (1974; 1976; 1977a; 1977b; 1979). In the first of these studies, using EPR, they investigated the effects of direct verses indirect damage upon dAMP. They concluded that indirect action (i.e. attack upon dAMP by 'OH or $\text{H}^-$) is not significant in the formation of nucleotide free radicals. They reached this conclusion by measuring total radical yields for $\gamma$-irradiated frozen solutions at different solute concentrations. It was discovered that upon increasing solute concentration the relative amount of 'OH produced upon irradiation begins to fall. They claimed, therefore, that the 'OH radicals formed were trapped specifically in the ice and that since the system was phase-separated these radicals could not interact with the nucleotide molecules. On warming, the 'OH radicals were found to disappear at ca. 135K, probably decaying by radical recombination (i.e. 'OH + 'OH $\rightarrow$ $\text{H}_2\text{O}_2$) leaving only dAMP free radicals. The experiment was extended to other nucleotides and the same results found. Thus, they concluded that on
\( \gamma \)-irradiation of frozen aqueous solutions of nucleotides directly damage processes only are involved.

Besides dAMP, other studies by Gregoli et al. upon TMP (termed dTMP) and 2'-deoxyguanosine-5'-monophosphate (dGMP) suggested a pattern of free radical formation in \( \gamma \)-irradiated frozen aqueous solutions of nucleotides. Direct ionisation of the nucleotide (N) appeared to give rise to electron-loss from the base to form a radical cation. The 'dry' electron then produced was found to add directly to the base to form a radical anion (Gregoli et al., 1974; 1976; 1977a). This is summarised in scheme 5.2.

**SCHEME 5.2**

\[
\begin{align*}
N & \xrightarrow{\gamma} N^+ + e^- \\
N + e^- & \rightarrow N^- \\
N^+ + H^+ & \rightarrow NH^+ \\
N^+ + \cdot OH & \rightarrow NOH^-
\end{align*}
\]

In each case the radical anion (or \( \pi^* \)-anion) was found to protonate at a carbon atom site (3). The radical cations (which may have been heteroatom deprotonated) were found to decay by one of two mechanisms. For the purines the radical cation was found to react with a hydroxyl ion, probably from the solvation water, to give an OH-adduct (4). For TMP it was claimed that the radical cation, not observed at 77K, deprotonates at the C5 methyl group to give a species known as T4 (2, X). They observed T4 at 77K and, on warming, found it to decay to be replaced by a quintet (c.f. chapter 2). The quintet was assigned to the hydroxylated radical cation ('TOH').

More recently the interpretations of Gregoli et al. (1976), concerning \( \gamma \)-irradiated frozen aqueous TMP, have been questioned (Hüttermann et al., 1992). Using EPR spectroscopy, Hüttermann et al. have analysed the free radical formation between 77K and 260K and concluded that nearly all are derived from reactions with electrons at
They further suggested that the primary electron-loss centre of TMP is not detectable at 77K, but that a minority species, ‘TOH (an EPR quintet), appears at 200K as a result of direct ‘OH addition to C6 of the thymine base - the ‘indirect’ damage pathway. They also noted the presence of an allylic radical at 77K (originally denoted T4 by Gregoli et al.) retained up to about 150K and demonstrated that this signal is considerably enhanced by the presence of H2O2. They concluded, therefore, that T4 is produced as a result of ‘OH-induced hydrogen atom abstraction from the methyl group at C5 - also the ‘indirect’ damage pathway.

The EPR spectrum for T4, an anisotropic quartet, was also detected in γ-irradiated low-temperature aqueous beryllium fluoride glasses - particularly for 6M BeF2/D2O containing 5-methylcytosine and warmed to 210K (Hüttermann et al., 1992). This is particularly interesting when compared to results for the present study. Using either γ-irradiated low-temperature aqueous 10M LiCl or 5.5 NaCl systems radical features very similar to those obtained by Hüttermann et al. for T4 are observed. However, the thermal stability of the LiCl glass is such that no positive identification of this radical can be made (chapter 2). For γ-irradiated frozen aqueous solutions, in either the presence or absence of 5.5M NaCl, warming to approximately 240K gives rise to a quintet (assigned previously as ‘TOH, Gregoli et al., 1976; Hüttermann et al., 1992). It may be coincidental but the second line of the quintet corresponds exactly with the feature assigned as part of T4 by Hüttermann et al. and with the feature observed in LiCl glasses. It is interesting to note that, in the present study, no such feature is observed until the complete decay of solvent radicals such as Cl2 and ClOH. Thus, due to the oxidising nature of the Cl2 radical it is possible that abstraction of an electron from a neighbouring thymine molecule may occur, i.e.

\[ T + \text{Cl}_2^{-} \rightarrow T^{+} + 2\text{Cl}^{-}. \]

The resulting radical cation may then deprotonate at the methyl group to give T4 or hydroxylate to give ‘TOH. The possibility that ‘OH radicals attack to give rise to both T4 and ‘TOH is certainly not ruled out for the LiCl and BeF2 systems. However, for the frozen aqueous and frozen aqueous sodium chloride systems it seems odd that T4 is only observed at temperatures between 77-150K, and that ‘TOH is only seen above 150K. Thus, although unlikely, a conversion from T4 to ‘TOH may have occurred as originally suggested by Gregoli et al. (1976).
Analysis of the products of γ-irradiated frozen aqueous solutions of DNA model compounds can assist in determining the radiation mechanisms involved (Sharpatyi et al., 1978; Cadet and Berger, 1985; Shaw, 1987; Shaw and Cadet, 1988; Shaw and Cadet, 1990). The γ-irradiation of frozen aqueous thymidine leads to a total of eighteen products not including isomers (Shaw, 1987). Of these, five are photoproducts - i.e. cyclobutithymidine dimers. Others, such as structure 5.I, suggest that T₄ is indeed formed and that it reacts with a neighbouring solute molecule to form a dimer. If T₄ were converted quantitatively into 'TOH we would probably not expect to see this molecule. Presumably, T₄ adds to the C6 position of the base of a nearby thymidine to form the radical 5.II. It is interesting that species 5.II has the same radical characteristics as those of 'TOH. Therefore, the EPR quintet, seen on decay of T₄, may well be due to 5.II and not 'TOH.

5.I

![Diagram of 5.I molecule]
In the present study both the nucleosides and nucleotides of cytosine and uracil, in particular dCMP and dUMP, have been considered along with those of thymine (chapters 3 and 4). The \( \pi^* \)-anions of cytosine (protonated at the exocyclic amino group) do not appear to carbon atom protonate upon warming, whereas most of the uracil \( \pi^* \)-anions do (at the C6 position to give the 5-yl radical). Indeed, for the latter, carbon atom protonation appears to take place in LiCl, NaCl and NaOH glasses as well as in the frozen aqueous system.

Photolysis (\( \lambda > 300\text{nm} \)) at 77K of \( \gamma \)-irradiated frozen solutions containing either thymine, cytosine or uracil compounds transforms the respective \( \pi^* \)-anions into hydrogen adduct radicals. Using EPR it is possible to monitor such a conversion from a \( \pi^* \)-anion doublet, for TMP or dUMP, into an octet or sextet respectively. For dCMP the poorly resolved triplet of the \( \pi^* \)-anion is replaced by the outer lines of a sextet. As for thymine, sextets for uracil and cytosine are believed to derive from C6 H-adducts, (or 5-yl radicals). Therefore, it appears that carbon atom protonation of the pyrimidine \( \pi^* \)-anions can be induced by photolysis. The case for cytosine is extremely interesting. Of the three pyrimidine \( \pi^* \)-anions only cytosine is known to be protonated. Evidence has been presented by previous authors that the others may be protonated but it is not conclusive. Thus, perhaps thermally-induced carbon atom protonation of the cytosine radical anion does not take place because the radical anion is already protonated, albeit at a heteroatom position. Whereas, for uracil and thymine, carbon atom protonation of the radical anions can occur since neither are already protonated.
Finally, studies on the products of γ-irradiated frozen solutions of 2'-deoxycytidine suggest that H-adducts are formed (Shaw, 1987). Six products are detected and all except two are uracil derivatives. Three of the uracil derivatives have the C5-C6 bond saturated, e.g. structure 5.III, suggesting a hydrogen adduct radical as an intermediate.

\[ \text{5.III} \]

5.1.2.2 DNA

Free radical formation in γ-irradiated DNA, as studied in the solid state by EPR, has attracted enormous interest since the earliest studies (Shields and Gordy, 1959; Shen et al., 1959; Boeg and Müller, 1959; Ehrenberg et al., 1963; Salovey et al., 1963; Pershan et al., 1964; Ormerod, 1965). Matrices available for studying DNA solid state radicals can be classified into three groups: powders, frozen aqueous solutions and oriented fibres. All these systems have been studied extensively over the years. In each case only DNA base free radicals are formed upon γ-irradiation at 77K. Work on oriented fibres, pioneered by Ehrenberg and co-workers (Ehrenberg et al., 1967; Gräslund et al., 1971; 1975; 1978), suggests only two primary species: one formed as a result of electron-loss and the other a result of electron-gain. The former, localised on the guanine base, gives a broad EPR singlet, whereas the latter, localised on the thymine base, gives an EPR doublet (Gräslund et al., 1971; Hüttermann et al. 1984).

Gregoli, Olast and Bertinchamps, following their early work on γ-irradiated frozen aqueous nucleotides, studied frozen mixtures of nucleotides and then DNA itself
They concluded that a long range electron transfer mechanism exists for DNA. This is strongly supported by a more recent study upon γ-irradiated frozen solutions of DNA containing intercalators whereby, on average, electrons are found to traverse 200 bases before becoming trapped (Callis et al., 1990). Gregoli et al. (1977b; 1979; 1982) demonstrated that the favourite sites of electron trapping in DNA are the pyrimidine bases. They concluded that, of these bases, cytosine has a greater electron-affinity than thymine. In a separate study, Sevilla and co-workers (1976; 1977) also demonstrated that the electron-affinity of the pyrimidines is greater than that of the purines but they concluded that those of cytosine and thymine are approximately the same. Therefore, electron trapping in DNA is expected to occur, at least to some extent, at cytosine as well as thymine. What led early workers to assume thymine as the major site of electron trapping in DNA is the formation of the 5,6-dihydrothymine-5-yl radical (TH). This radical, discussed in detail in chapter 2, can be unambiguously assigned due to its EPR spectrum. It is observed in oriented fibre studies (Graslund et al., 1975), frozen aqueous solutions (Gregoli et al., 1982; Boon et al., 1984) and lyophilised powders (Hüttermann et al., 1992). On warming these systems the TH octet grows-in as the T doublet decays.

More recently, a complete re-evaluation of the primary electron trapping site within DNA has taken place. Bernhard (1989b; 1990) found cytosine to be favoured over thymine as the site of electron trapping in X-irradiated DNA oligomers at 4K. As discussed in chapter 3, electron addition to cytosine, involved in base pairing or base stacking, probably gives rise to the N3-protonated radical anion (an EPR doublet). Using the tetramer d(pGpCpGpC) in 12M LiCl to produce a 'bench-mark' doublet for the cytosine radical anion, as it might appear in γ-irradiated DNA, Bernhard has demonstrated that 80% of the electrons add to cytosine in the DNA tetramer d(pApGpCpT). Only 15% electron addition to thymine was found. Similar results have been obtained by Sevilla et al. (1991; 1992) for γ-irradiated single- and double-stranded DNA. Using 'bench-mark' EPR doublets for the one-electron adducts of both cytosine and thymine, Sevilla and co-workers have found that 77% of electrons add to C, and only 23% add to T for double-stranded DNA. The relative abundances of electron adducts in single stranded DNA, on the other hand, favours T slightly (60-70% on T and 30-40% on C). Furthermore, they have discovered that greater than 90% of positive holes end up on the guanine base for double-stranded DNA, which drops to approximately 60% for single-stranded DNA.
In most DNA studies the electron-gain or -loss centres are referred to as pristine radical ions. In reality, as was demonstrated for the electron adducts of monomeric cytosine derivatives, the radical anions are probably heteroatom-protonated and the radical cations heteroatom-deprotonated. EPR shows that the source of the proton for \( C^- (H^+) \) in DNA is likely to be the neighbouring hydrogen-bonded guanine base, figure 3.IX. There is no clear EPR evidence that \( T^- \) is protonated in either thymine derivatives or DNA itself (chapter 2). However, due to the facile nature of heteroatom-protonation of \( C^- \) (see chapter 3), it is possible that \( T^- \) may also protonate. If so, for \( T^- (H^+) \) in DNA, the likely source of a proton is the surrounding solvation water. It should be noted that the simple reaction,

\[
T^- + H_2O \rightleftharpoons T^-(H^+) + \cdot OH,
\]

would probably favour the reverse process and thus a chain of water molecules is required, scheme 5.3. This would remove the \( \cdot OH \) ion from the vicinity of the \( T^- (H^+) \) unit to form a remote \( X^- \) anion (any of the more acidic protons in the neighbourhood should suffice). Loss of solvating water molecules from the vicinity of the DNA will break the chain and inhibit \( T^- (H^+) \) formation.

**SCHEME 5.3**

EPR/ENDOR evidence from studies of X-irradiated purine single crystals suggests that, for DNA, the radical cation of guanine is deprotonated at 77K (Nelson et al., 1992). Furthermore, Nelson and co-workers in an extensive study of adenine and guanine single crystals have found that the primary oxidation product in each case deprotonates rapidly. The site of deprotonation is dependent upon the environment for each crystal and they conclude that, for DNA, no definitive assignment of the site of

99
deprotonation can be made. Further support for the idea that G⁺ may well be heteroatom deprotonated is presented by Steenken (1989). He shows that the pKₐ of guanine in the C-G base-pair is about 9.4 and that removal of an electron from G to give G⁺ greatly increases the acidity (pKₐ of G⁺ is 3.9). Thus, G⁺ in double-stranded DNA is likely to give up a proton to its hydrogen bonded cytosine neighbour. Similarly, for the A-T base-pair, deprotonation of the radical cation of adenine in DNA should be extremely facile due to its acidic nature (pKₐ ≤ 1).
5.2 EXPERIMENTAL

5.2.1 Materials

Type I sodium salt calf-thymus DNA was obtained from BDH Chemical Company. Hydrogen peroxide, pyrogallol (1,2,3-trihydroxybenzene), potassium hydroxide pellets and anhydrous copper(II) sulphate were obtained from Aldrich Chemical Company. All other materials were supplied as described in chapters 2, 3 and 4.

5.2.2 Methods

5.2.2.1 Experimental

Due to the reactive nature towards ambient oxygen, on warming, of the free radicals produced by the γ-irradiation of frozen aqueous DNA, all residual oxygen was 'scrubbed' from the nitrogen gas prior to de-gassing. The 'scrubber' consisted of a 1M solution of pyrogallol in 1M potassium hydroxide in a series of Dreschel bottles. For each bottle 50g of KOH and 15g of pyrogallol in two 50ml aliquots of distilled water were used. These were then de-gassed thoroughly before mixing. The fresh solution was pale yellow and turned black when saturated with oxygen.

DNA solutions were made up as 50mg of DNA in 1ml of doubly distilled/deionised water and stored at 4°C for 48 hours to allow the DNA to fully dissolve. For the experiment involving hydrogen peroxide, H₂O₂ was dissolved in the distilled water, to the required concentration, prior to the addition of DNA. Deoxygenation (or de-gassing) of the DNA solution(s) was carried out in a nitrogen 'tent' by bubbling oxygen-scrubbed nitrogen gas through each sample for 10 minutes at a pre-set flow rate. TMP, at a concentration of 250mg ml⁻¹, was also deoxygenated for 10 minutes at the same pre-set flow rate. Each sample was then frozen into pellets in liquid nitrogen as described in chapter 2. The DNA pellets were individually labelled on
lengths of cotton before being placed into an irradiation jar. Pellets were then irradiated in the same jar so that a consistent dose could be obtained. Irradiations were achieved by use of a Vickrad 90Co γ-ray source with a dose of approximately 1Mrad (TMP frozen pellets were also given a dose of approximately 1Mrad). After irradiation, the samples were removed from the irradiation jar and their labels removed. EPR spectra were recorded against the standard [Fe(acetylacetonate)3, which has an EPR absorption at g = 4.34, for Varian, or MnO for Jeol] at 77K using a field modulation of 4G. Relative intensity measurements were more easily made on the Jeol spectrometer since it was possible to 'dial' the MnO marker to a specific depth in the cavity (whereas the Fe(acac)3 marker had to be placed inside the finger Dewar and held vertically on a wire next to the pellet).

Subsequent to this measurement the samples (DNA or TMP) were placed in specially designed annealing tubes. These were then inserted into a copper block cryostat (a cylinder of solid copper with 16 holes drilled vertically at one end) which was able to hold up to 15 samples and the thermocouple. The copper block was cooled in a similar manner to that used for the study of frozen beads (chapter 2). Each sample was left at the required temperature for exactly 7 minutes to equilibrate, then re-cooled to 77K for the next EPR measurement to be made.

5.2.2.2 Isolation of elementary EPR patterns for DNA

The EPR spectrum of γ-irradiated frozen aqueous DNA (50mg ml-1), warmed to 130K to remove OH radicals from the ice phase, consists of a broad singlet (figure 5.1). Previously, it has been shown that this singlet consists of an electron-loss centre [i.e. G+/G+(-H+)] and possibly A+/A+(-H+), both unresolved singlets] and an electron-gain centre [i.e. T'/T'(H+) and C'(H+), both nearly identical doublets], Sevilla et al., 1991. For simplicity, in future, T'/T'(H+) will be referred to as T−, C'/C'(H+) as C−, G+/G+(-H+) as G− and A+/A+(-H+) as A−. In order to make quantitative assessments of DNA free radical populations it is necessary to isolate these species and produce elementary EPR patterns for each.

On warming, in the absence of oxygen, an octet corresponding to the TH radical is observed to grow in. This is formed by the C6-protonation of T−. The fate of both C− and G− is as yet unknown. In the presence of oxygen all EPR features are overtaken by the formation of peroxy radicals (ROO·). The EPR spectrum of ROO· is a very characteristic anisotropic singlet which is virtually uninfluenced by changing the
Figure 5.1. EPR spectrum of a γ-irradiated frozen aqueous solution of DNA (50mg ml⁻¹), warmed to 130K to remove 'OH radicals and recorded at 77K at low power (0.01mW). It consists of a broad singlet assigned to a mixture of electron-loss and -capture centres from the purine and pyrimidine bases respectively.
Figure 5.2. EPR spectrum of a γ-irradiated frozen aqueous solution of DNA (50mg ml⁻¹), warmed to 130K and recorded at 77K at high power (0.1mW). Due to saturation of the pyrimidine π*-anion doublet this spectrum is rich in the π-cation
ROO' concentration is found to vary depending upon the concentration of oxygen in the frozen aqueous sample (Gregoli et al., 1982; Boon et al., 1984).

Gregoli et al. (1982) have demonstrated that a variety of experimental spectra, obtained from irradiated samples of DNA under a range of conditions (e.g. DNA hydration, base composition and oxygenation), can be simulated by linear combinations of the four elementary patterns (G*, T*, TH and ROO'). The present study has attempted to use this methodology but with certain modifications, i.e. the doublet generated also represents the C' content within the γ-irradiated DNA system. Furthermore, since oxygen has been removed from the samples, the ROO' pattern is not expected to contribute to the spectra. Isolation of the three patterns (G*, [T* + C'] and TH) was carried out using the methods described by Gregoli et al. (1982), Jones (1987) and McClymont (1990).

As already mentioned, figure 5.1 represents the EPR spectrum of a γ-irradiated deoxygenated solution of DNA, annealed to 130K to remove the OH radical signal and recorded at 0.01mW. The spectrum at a higher microwave power (0.1mW) is shown in figure 5.2. Making the assumption that the low-power spectrum contains approximately equal amounts of electron-loss and electron-gain centres, whilst the one at higher power partially saturates the T*+ C' signal, it is possible to obtain a 'pure' EPR spectrum for G+. This is achieved by careful subtraction of small amounts of the low-power spectrum from that at high-power, and relies on the fact that the doublet spectrum saturates more readily than the G+ singlet (Sevilla et al., 1991). Using the G+ singlet the reverse subtraction now generates an EPR doublet, figure 5.3, corresponding to T*+ C' (henceforth referred to as Py'). To check the accuracy of such spectra the doublet was compared to that produced for DNA in a 10M LiCl glass (Malone, 1991). The G+ singlet was compared to that generated by Gregoli et al. (1982). A good match was obtained in each case. The power saturation method is a good way of producing the Py' doublet since it does not require the use of model compounds which, as shown in chapters 2 and 3, can give rise to different results depending upon environment.

A 'pure' spectrum for TH was generated using the method of McClymont (1990). A relatively 'dry' sample of DNA (approximately 10% H2O by volume) was allowed to equilibrate over a saturated solution of cupric sulphate for 48 hours. The sample of DNA, now at 100% humidity, was frozen, irradiated, annealed to ~270K and then observed by EPR at 0.01mW. An eight-line spectrum was detected which, on
Figure 5.3.
Elementary EPR patterns for:
(a) $G^+/G^+(-H^+)$ and possibly $A^+/A^+(-H^+)$, i.e. $Pa^+/Pa^+(-H^+)$,
(b) $T^+/T^+(H^+)$ and $C^+/C^+(H^+)$, i.e. $Py^+/Py^+(H^+)$,
(c) TH.
subtraction of small amounts of $G^+$, compared favourably with that of Gregoli et al. (1982), figure 5.3.
5.3 RESULTS AND DISCUSSION

5.3.1 Gamma-irradiated frozen aqueous DNA

5.3.1.1 The utilisation of DNA elementary patterns

Using the procedure outlined in the experimental section, EPR spectra for irradiated frozen aqueous DNA (50mg ml\(^{-1}\)) have been obtained over the temperature range 77K-240K, figure 5.4. At 77K a strong signal from hydroxyl radicals is observed, making it difficult to evaluate the relative contributions of electron-loss and electron-gain centres. Clearly no TH is present at 77K, which contrasts with aqueous low-temperature glasses and frozen aqueous TMP (chapter 2) and suggests that hydrogen atom addition to the C6 position of thymine does not take place for frozen aqueous DNA.

On warming to 130K the hydroxyl radical signal is removed to reveal an unresolved EPR singlet. This singlet, which was used originally to obtain the 'pure' EPR patterns for the electron-loss and -gain centres, can be matched against a library of doublet + singlet (i.e. Py\(^-\) + G\(^+\)) spectra. The library was constructed on computer in 1% steps from 100% G\(^+\) (the singlet) to 100% Py\(^-\) (the doublet). Not surprisingly, since this spectrum has been used as standard for doublet + singlet, a 50:50 fit is obtained. It is likely that the actual relative percentages are not quite equal (c.f. Sevilla et al., 1991). It should be noted that at 130K a small quantity of TH is observed (approximately 2%). This was originally subtracted prior to the computer subtractions of the high- and low-power DNA to produce 'pure' EPR patterns.

By double integration of the EPR spectrum obtained at 135K, a 100% free radical population standard was obtained. All values are given relative to this standard, table 5.1. The EPR spectra recorded for temperatures of 165K and above all contain TH to varying degrees. In each case, TH was subtracted out on computer and the resulting spectrum analysed for relative contributions of singlet (G\(^+\)) and doublet (Py\(^-\)). The results are represented both in table 5.1 and graph 5.1. It can be seen that TH
Figure 5.4. EPR spectrum of a γ-irradiated frozen aqueous solution of DNA (50mg ml⁻¹), showing a broad singlet attributed to both DNA electron-loss and capture centres and features from OH radicals trapped in the ice-phase.
Figure 5.4 contd.. EPR temperature profile of a γ-irradiated frozen aqueous solution of DNA (50mg ml⁻¹), showing the loss of the broad singlet attributed to both electron-loss and -capture centres and a concurrent growth of TH. All spectra recorded at 77K.
### TABLE 5.1
Percentage contributions of the elementary EPR patterns to a range of DNA spectra

<table>
<thead>
<tr>
<th>TEMPERATURE /K</th>
<th>TOTAL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>G&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Py&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TH&lt;sup&gt;c&lt;/sup&gt;</th>
<th>TOTAL - TH&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>130</td>
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<td>8.6</td>
<td>76.0</td>
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<td>18.0</td>
<td>19.2</td>
<td>43.0</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
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<td>9.0</td>
<td>-</td>
<td>-</td>
<td>3.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estimated error = ±5%, based on the measurements of double integrals for 10 samples.

<sup>b</sup> Estimated error = ±3%, based on visual comparison with a library of computed spectra - see text.

<sup>c</sup> Estimated error = ±2%, based on the measurements of double integrals for 10 samples.
Graph 5.1. Variation with temperature of radical populations for gamma-irradiated frozen aqueous DNA (50mg/ml)
Graph 5.2. Variation with temperature of radical populations for gamma-irradiated frozen aqueous TMP (0.25M)

Temperature/K

Total Spins (%)
steadily grows in, reaching a maximum value at approximately 190K of about 19%.
Thus, assuming an equal contribution of electron-loss and -gain centres at 130K, it is
reasonable to suggest that at least 38% (±2%) of Py⁻⁻ is converted to TH.

SCHEME 5.4

\[
\begin{align*}
C⁻⁻(H⁺) & \rightleftharpoons C⁻ + H⁺ \\
C⁻ & + T & \rightleftharpoons C + T⁻ \\
T⁻⁻ & + H⁺ & \rightarrow TH
\end{align*}
\]

If both anionic centres are initially protonated then the probability of thermally-
induced electron transfer between C and T bases in DNA is likely to be low. Thus,
providing TH is only formed by C6-protonation of the thymine \( \pi^* \)-anion, then the
relative yields of TH radicals can be used as a measure of the ratio of C⁻⁻ to T⁻⁻. On
this basis, no more than 64% of electrons can be trapped at cytosine at 130K. This
result is at variance with those of Bernhard (1989b) and Sevilla et al. (1991). The
former suggests a value of about 90% electron addition to cytosine in DNA oligomers,
whilst the latter claims a value of 73% for frozen aqueous DNA. In order to reconcile
these results it is necessary to postulate the thermally-induced electron-transfer
mechanism from C⁻⁻(H⁺) to T. If such a mechanism does occur then it simply
introduces a more complicated route to the 64% value and makes it impossible to assess
the relative contributions of the one-electron adducts at 130K. Step (1), scheme 5.4,
requires the loss of a proton from C⁻⁻(H⁺) which, since C⁻⁻ has a very high proton
affinity, should have a low probability. Step (2) occurs in the primary ionisation stage
and presumably involves considerable movement of the electron. Step (3) is expected to
be relatively slow since protonation must occur through the neighbouring solvation
water molecules and not the base-pair hydrogen bonding network.

One question arising from thermally-induced electron-transfer from C⁻⁻(H⁺) to
T is that, if it occurs, why is only a 38% overall conversion of doublet to octet
observed? It should be noted that this value is a minimum since TH begins to decay at
around 180K (Malone, 1991). Furthermore, if an electron becomes mobile upon warming there is a high probability that it might return to a $G^+$ site thereby giving rise to thermoluminescence. There are no reports in the literature of thermoluminescence in the required temperature range.

Careful computer subtraction of 'TH quantities from EPR spectra over the temperature range 130K to 240K results in the spectra shown in figure 5.5. It is possible to match the spectra at 130K, 165K and 190K with those of the computer simulated additions $[G^+ + Py^-]$, table 5.1. However, above 190K the subtracted spectra do not resemble any of the computer mixtures. These spectra may well represent new radical species, i.e. from the sugar moiety (see section 5.3.2). However, it should be noted that computer subtractions of EPR spectra can sometimes produce artifacts.

5.3.1.2 Comparison of results for DNA with TMP

Using the procedures outlined for DNA, it is possible to estimate the relative percentage conversion of $T^+T^-(H^+)$ to 'TH for $\gamma$-irradiated frozen aqueous TMP. As discussed earlier, Gregoli et al. (1976) suggested that equal amounts of electron-gain and -loss centres are observed on warming a $\gamma$-irradiated frozen aqueous sample of TMP to 130K. Hüttermann et al. (1992) disagreed with this observation and suggested that nearly all the radicals observed at 130K are due to electron-gain centres. In the present study, in order to assess both suggestions, a sample has been warmed and the relative growth of 'TH monitored, graph 5.2. Unlike with DNA, 'TH reaches a maximum value at 225K. This value is approximately 44% ($\pm1$) of the original total radical population at 130K. Thus, if equal contributions of electron-loss and -gain centres are assumed at 130K, then at least 86% are converted to 'TH. Even if the majority of the free radicals are attributed to electron-gain then at least 43% are converted into 'TH - more than for DNA.

The relative stability of 'TH in TMP over that of 'TH in DNA suggests different mechanisms of decay in each case, which is probably a result of the different radical environments. TMP undergoes base-stacking in frozen aqueous solution (Ts'0 et al., 1974). As with DNA, a solution of TMP gives rise to two phases upon freezing - ice and a glass-like region. The latter is responsible for transferring initial damage centres, i.e. positive 'holes' or electrons to DNA or TMP. For Hüttermann et al., (1992) to be
Figure 5.5. EPR temperature profile of a γ-irradiated frozen aqueous solution of DNA (50mg ml⁻¹) with TH subtracted out. The spectra show the 'normal' broad singlet (assigned to electron-loss and -capture centres) up to 190K. Beyond 190K features of a new species, tentively assigned to sugar radicals, are observed.
correct the positive holes must be fixed and thereby trapped in this phase (or close to it) - c.f. aqueous LiCl glasses. Certainly, the present study demonstrates that this cannot be true for DNA where roughly equal amounts of electron-loss and -gain centres have been identified.

5.3.2 The UV-photolysis of a frozen aqueous solution of DNA containing hydrogen peroxide

5.3.2.1 Intramolecular H-atom abstraction

A possible mechanism for TH decay in DNA is via intramolecular hydrogen-atom abstraction from a nearby deoxyribose moiety (Symons, 1987). This would result in the formation of a sugar radical species, figure 5.6, which might ultimately give rise to strand scission i.e. a single strand-break, scheme 5.5.

**SCHEME 5.5**

\[
\text{BASE}^+ + \text{C-H} \rightleftharpoons \text{BASE-H} + \text{C.} \quad - \text{(1)}
\]

\[
\text{C.} \xrightarrow{\text{fast}} \text{BREAK-DOWN PRODUCTS} \quad - \text{(2)}
\]

Step (1) is probably endothermic. It requires that the C-H hydrogen atoms are 'poised' close to the reaction centre and that, after transfer, the sugar radicals can react further forcing the equilibrium to completion, step (2).

5.3.2.2 The generation of sugar radicals

Hydrogen peroxide has been used previously as an additive for frozen aqueous DNA (Cullis et al., 1986). This study demonstrated that, on warming to ca. 195K,
Figure 5.6. A pyrimidine trinucleotide fragment (CTC) of B-DNA. The thymine base consists of the TH radical and the distances to the nearest five hydrogen atoms (from the neighbouring sugars) that may be candidates for hydrogen atom abstraction by TH are indicated by arrows.
'OH radicals were able to abstract hydrogen atoms from deoxyribose units to give rise to sugar radicals from DNA. No features attributable to sugar radical species were detected in the absence of H$_2$O$_2$. Thus, they concluded that, in the absence of additives, 'OH radicals are generated in the ice phase, trapped and react only within this phase upon warming.

A problem arising from γ-irradiation of frozen solutions of DNA containing H$_2$O$_2$ is the EPR observation of an ice-phase 'OH radical signal at 77K. However, using light from a high-pressure mercury lamp (mainly 313 + 365nm) such formation cannot occur. This light only gives rise to homolytic bond fission of H$_2$O$_2$ molecules, which are almost certainly confined to the 'glassy' region, and generates 'OH radicals. Since these are now trapped in the 'glassy' region they can react either with DNA or with themselves. It should be noted that the UV-irradiation of frozen aqueous DNA in the absence of H$_2$O$_2$ generates no significant DNA damage.

In the presence of H$_2$O$_2$, an EPR signal in the free-spin region is observed, figure 5.7. At high microwave powers it consists of a low-field doublet characteristic of HO$_2^-$ radicals. At low-microwave powers, however, a broad singlet together with a poorly defined 15G sextet are detected. HO$_2^-$ is probably formed by reaction of 'OH with a neighbouring H$_2$O$_2$ molecule, i.e.

\[
H_2O_2 + 'OH \rightarrow HO_2^- + H_2O
\]

The low-power species are likely to be derived from 'OH attack upon DNA which, despite liquid-phase results indicating preferential addition of 'OH to the DNA bases, seems to give rise to sugar radicals. EPR spectra for most of the possible 'OH radical-DNA base adducts have been reported in the literature (Gregoli et al., 1974; 1976; 1977a), but none are observed here. Furthermore, sugar radical formation has been observed for frozen solutions of DNA containing iodoacetamide (Cullis et al., 1985), but the EPR spectra are somewhat complex making it difficult to compare to the present results. The spectra seen upon computer subtraction of 'TH (section 5.3.1) may also be sugar derived, but do not compare with the H$_2$O$_2$ results.

In conclusion, this experiment is further proof that for frozen aqueous DNA, in the absence of additives, 'OH radicals, generated by γ-irradiolysis, must be formed and trapped in the ice phase. Thus, they cannot react with DNA upon warming. Certainly,
Figure 5.7. EPR spectra of a UV-irradiated frozen aqueous solution of DNA (50mg ml⁻¹) containing hydrogen peroxide (40mM). The spectra recorded at 77K (a) at low power showing features assigned to sugar radicals and (b) at high power showing features assigned to the HO₂⁻ radical.
if 'OH radicals were generated in the DNA 'glassy' region then it is reasonable to suppose that the species observed here should then be detected.
5.4 CONCLUSIONS

A frozen solution of DNA consists of two separate phases, as originally postulated (Gregoli et al., 1982; Cullis et al., 1986). On γ-irradiation all damage that occurs within the ice-phase (i.e. OH) is confined therein - as observed by EPR spectroscopy. Upon warming, the OH radicals react within this phase. They do not react with the DNA. DNA, which is in a separate glass-like region, undergoes 'direct' damage upon irradiation. Positive 'holes' and electrons generated within this region are transferred to the polymer and ultimately trapped.

Originally, trapping was believed to occur specifically at two base sites: Electron-loss centres confined to guanine (G⁺) and those of electron-gain to thymine (T⁻), Symons (1987). It was suggested that these centres could lead ultimately to a pair of single strand-breaks which, if formed close together, would give rise to a double strand break. It now seems likely that this G⁺/T⁻ model was an over-simplification. Electron trapping almost certainly occurs at the cytosine base as well as at thymine.

Electron migration in γ-irradiated frozen aqueous DNA has been shown to be extensive though the use of intercalators (Cullis et al., 1990). A major factor in electron trapping in these solid state systems is the relative electron affinities of the DNA bases and or DNA additives, i.e intercalators, nitroimidazoles >> cytosine ≈ thymine > adenine ≥ guanine (Sevilla, 1977; Gregoli, 1979b; Boon et al., 1985; Cullis et al., 1990). However, when the electron-affinities are relatively close together trapping is probably influenced by proton-transfer which, unless it is reversible, should completely quench further charge migration. Thus, the extent of electron trapping at either pyrimidine is possibly determined by heteroatom protonation of the radical anions. As discussed in chapter 3, protonation of C⁻ is likely to take place from the N1 proton of guanine, which is hydrogen bonded to N3 of cytosine in the C-G base-pair. If heteroatom protonation of T⁻ occurs, it would be expected from the surrounding solvation water. Thus, protonation of C⁻ is likely to be more facile than that of T⁻.

On this basis it might be expected that all electrons should be trapped at C rather than T. However, there can be little doubt that trapping at both sites takes place (Sevilla
et al., 1991; 1992; Malone, 1991). Furthermore, unless a thermally-induced electron-transfer mechanism is invoked, at least 36% of electrons are trapped at T as monitored by the C6-protonation of the thymine π*-anion (i.e. TH formation). Thus, the factors affecting such trapping must be extremely subtle and that it may be quite possible to alter the ratio of electron adducts by, for example, reducing the water content around the DNA. Indeed, results for oriented DNA, where the concentration of water is considerably reduced below the 100% humidity value, are quite definitive in showing that T' is a minor component (Hüttermann et al., 1992). These results can most easily be interpreted in terms of the relative protonation rates for C" and T". In duplex DNA, the degree of hydrogen bonding for cytosine, particularly at N3, within the G-C base-pair, is independent of water concentration, whereas hydration of the O4 site of thymine is expected to decrease as water is removed. Thus, C"(H+) would increase relative to T"(H+) as the water content decreases.

Finally, a question arises as to the fate of C"(H+) on warming. If it does not lose its electron and return to the parent base then presumably it can undergo some form of carbon atom protonation to give CH - similar to that of T" to give TH. The present results for dCMP (chapter 3) demonstrate that a hydrogen-adduct of cytosine can be formed upon the photolysis of the π*-anion, giving rise to an EPR sextet. Although no spectrum of this kind is observed for γ-irradiated frozen aqueous DNA, it does not mean that CH is not formed in DNA. It may be too short-lived to be detected. Future work might perhaps concentrate upon product analysis of DNA, or oligomers, with particular reference to cytosine which has been saturated at the C5-C6 bond. If such products were found it would strongly suggest that C"(H+) gives rise to 'CH rather than give up its electron to thymine.
Appendix

Electron Paramagnetic Resonance Spectroscopy (EPR)
A.1 INTRODUCTION

Electron Paramagnetic Resonance Spectroscopy (EPR), also known as Electron Spin Resonance Spectroscopy (ESR), is mainly used for the study of molecules containing one or more unpaired electrons. Since the technique was discovered by Zavoisky (1945) it has provided detailed structural information on numerous paramagnetic systems. The basic principles of EPR, briefly described here, are covered in detail by several excellent texts (Poole, 1967; Wertz and Bolton, 1972; Atherton, 1973; Symons, 1978).

Much of the knowledge of the structure of molecules has been obtained from molecular absorption spectra whereby attenuation verses wavelength (or frequency) of a beam of electromagnetic radiation is measured as it passes through a sample of matter. Spectral lines or bands are produced which correspond to transitions between energy levels of a molecule, and the frequency of each is a measure of the energy difference between two levels. Electromagnetic radiation can be regarded as coupled electric and magnetic fields, oscillating perpendicular to one another and to the direction of propagation (figure A.1). Either component can interact with molecules.

In most cases it is the electric field component which interacts with the molecules and for absorption to occur the energy of a quantum must correspond to the separation of energy levels in the molecule. In addition to this, the oscillating electric field component must stimulate an oscillating electric dipole in the molecule. Thus, molecular rotation of a molecule containing a permanent electric dipole moment, e.g. HCl, will give rise to an absorption.

It might be expected that a molecule containing magnetic dipoles might interact with the magnetic component of electromagnetic radiation. However, irradiation of such a molecule over a wide range of frequencies usually produces no absorption attributable to a magnetic interaction. Only when the sample is placed in a static magnetic field absorption attributable to magnetic dipole transitions will occur at one or more characteristic frequencies. The requirement of a static magnetic field, which may
Figure A.1. Instantaneous amplitudes of electric and magnetic field components in a propagating electromagnetic beam.
be contributed to internally by the nuclei of a molecule, is the unique aspect of magnetic dipolar transitions.

Permanent magnetic dipoles in a molecule are associated either with electrons or with nuclei and arise from net electronic or nuclear angular momentum. Magnetic dipoles attributable to electrons arise from net spin or net orbital angular momenta, or from a combination of the two. In the vast majority of cases over 99\% of the magnetic dipole is due to spin angular momentum with a small orbital contribution. The magnitude of this spin angular momentum, denoted $p_s$, arising from rotation of the electron about its own axis, is given by

$$p_s = \sqrt{s(s+1)} \frac{h}{2\pi}$$  \hspace{1cm} (A.1)

where $s$ = spin angular momentum quantum number ($s = \frac{1}{2}$ for an electron) and $h$ = Plank's constant. Quantum theory postulates that spin angular momentum of electrons is quantised. Therefore, the magnetic moment arising from spin angular momentum of an electron is also quantised. The magnitude of the magnetic moment associated with the spin of an electron, $\mu_s$, is given by

$$\mu_s = \frac{-e}{m_ec} p_s$$  \hspace{1cm} (A.2)

where $e$ = electronic charge, $m_e$ = mass of electron and $c$ = velocity. When an electron is placed under the influence of an externally applied magnetic field it tends to align itself with its magnetic moment vector along the field direction (z-direction or z-axis), i.e. it behaves as a magnetic dipole. Due to the fact that angular momentum is quantised, the spin angular momentum vector, with magnitude $p_s$, may take up certain orientations with respect to the z-axis such that the component $(p_s)_z$ along the z-axis is given by

$$(p_s)_z = m_s \frac{h}{2\pi}$$  \hspace{1cm} (A.3)

where $m_s$ = magnetic spin quantum number, which defines possible values of the z-axis component of the spin angular momentum of the electron. Quantisation of the spin angular momentum corresponds to a finite number of possible orientations of the
electron with respect to an externally applied magnetic field. Each orientation represents a discrete energy level and transitions between these levels may be induced by interaction with electromagnetic radiation.

A.2 BASIC THEORY

A.2.1 Introduction

For a single unpaired electron, which is only influenced by an external magnetic field and whose magnetic moment (μₚ) is entirely due to its spin (ℏ/2), there are two possible orientations. The magnetic quantum numbers corresponding to these orientations are denoted as mₑ = -ℏ/2 and mₑ = +ℏ/2, figure A.2. If an oscillating magnetic field, provided by microwaves of fixed frequency, is applied perpendicular to the static magnetic field (denoted H₀), transitions between the energy levels can occur. This is provided the energy of the photon for the oscillating magnetic field is equal to the energy difference of the two levels, i.e.

\[ hν = 2μₚH₀ \]  

(A.4)

Such transitions between energy levels are possible for Δmₑ = ± 1. Since the spin magnetic moment of an electron is known, 1.0011596ₚ (Sommerfield, 1957; Wilkinson and Crane, 1963), where β is the Bohr magneton, then for a free electron transition can occur when

\[ hν = 2.00232βH₀ \]  

(A.5).

The value 2.00232 is called the electron free spin g-factor (or gₑ). Equation A.5, which illustrates the fact that this is a resonance phenomenon, is true for a free electron. In general, however, the unpaired electron will be part of a radical or trapped in some way such that it is subject to magnetic interactions other than the external magnetic field. Thus,

\[ hν = gβH₀ \]  

(A.6)
Figure A.2. The effect of an applied static magnetic field ($H_B$) on the $m_s = \pm \frac{1}{2}$ levels of an unpaired electron. These are degenerate at zero field. The thick lines represent the spin-only behaviour, the transition ($h\nu$) occurring at a field corresponding to $g_0 = 2.0023$. The dashed lines are for a radical having a low-lying, vacant excited state, giving $g_1 < 2.0023$, and the dotted lines are for a radical having a neighbouring filled level, giving $g_2 > 2.0023$. 

$\mu_B$, $g_0$, $g_1$, $g_2$.
where \( g \) has values other than 2.00232, figure A.2.

### A.2.2 The absorption of power

At frequencies used in EPR, absorption and emission, corresponding to transitions up and down energy levels, are equally likely to occur. Thus, for a net absorption of radiation to be observed there must be a difference of population between the number of unpaired electrons in the two states. In the absence of radiation, populations are determined according to the Boltzman distribution. Therefore, the population difference is described by

\[
\frac{N_- - N_+}{N_- + N_+} = \frac{e^{\mu H_0/kT} - e^{-\mu H_0/kT}}{e^{\mu H_0/kT} + e^{-\mu H_0/kT}}
\]  

(A.7)

where \( N_- \) and \( N_+ \) are the number of spins (i.e. unpaired electrons) in the sample in the lower and upper energy levels, \( k \) is the Boltzman constant and \( T \) the absolute temperature. If the total number of unpaired electrons is denoted \( N \) and the population difference between the two levels denoted \( n \) then

\[
n = N \tanh(\mu H_0/kT)
\]  

(A.8)

where the ratio \( n/N \) is called polarisation. Under normal EPR working temperatures (i.e. 77K or room temperature) \( \mu H_0/kT \) is very small and thus,

\[
n = N \frac{\mu H_0}{kT} = \frac{Ng\beta H_0}{2kT}
\]  

(A.9)

From equation A.9 it can be seen that sensitivity is improved by going to higher magnetic fields and to lower sample temperatures.

If the unpaired electrons in the sample were to interact only with the magnetic field and the microwave radiation, then the effect of the microwave radiation would be to make the populations in the two levels the same; hence the absorption would cease. However, the unpaired electrons can, in fact, change from one level to another without assistance from microwave radiation by 'relaxation processes'.
When the microwave power used is too large a signal can 'saturate' which causes a loss of line intensity due to equalisation of energy level populations. As shown in chapter 5, this phenomenon can be very useful if a sample contains two radical species, i.e. Pu$^+$ and Py$^-$. Py$^-$ saturates at lower microwave powers than Pu$^+$ and thus it has been possible to isolate both spectral components.

A.2.3 The g-factor

The g-factor is defined as a constant in equation A.6 such that

$$g = \frac{\hbar v}{\beta H_0}$$

(A.10)

It is a characteristic quantity of the environment of the unpaired electron and its measurement assists in the interpretation of an unknown radical species. It is a tensor, characterised by three principal g-factors, $g_{xx}$ (often denoted $g_x$), $g_{yy}$ (or $g_y$) and $g_{zz}$ (or $g_z$). These are defined along the principal axis of the group containing the unpaired electron. In a single crystal the g-factors can be measured by applying a magnetic field along the principal axes of the molecule. At intermediate orientations the g-factors depend upon the angle $\theta$. For an axially symmetric system, where $g_x = g_y \neq g_z (g_x + g_y$ is often denoted as $g_1$, and $g_z$ as $g_3$), then the g-factors will vary with magnetic field orientations as

$$g(\theta)^2 = g_1^2 \cos^2 \theta + g_3^2 \sin^2 \theta$$

(A.11)

It should be noted that, by convention, $g_3$ is defined as the g-factor observed when the applied field is parallel to the symmetry axis, hence the term $g_{11}$ is the value when the applied field is perpendicular to the symmetry axis.

When the species containing an unpaired electron has symmetry with equivalent principal axes, i.e. a cube, tetrahedron or octahedron, then $g_x = g_y = g_z$ and the g-factor is said to be isotropic. In a frozen sample molecules will be randomly orientated. This gives rise to a spread of g-factors, now said to anisotropic, comprising individual absorptions of molecules. The anisotropy of a spectrum's g-factors will be averaged in a sample, particularly liquids, if the molecular motions are sufficiently rapid.
A.2.4 Nuclear hyperfine interactions.

Magnetic interactions of unpaired electrons with surrounding nuclei give rise to hyperfine structure. This interaction can either be of a type which is isotropic and allows delocalisation of the unpaired electron onto the nucleus, or a dipolar interaction between the spins of the electron and nucleus (which is anisotropic). For the isotropic hyperfine interaction the nuclear spin produces a local field at the electron and, in general, there are \(2l + 1\) orientations of the nuclear spin in a magnetic field, each corresponding to a different energy. These orientations correspond to an EPR spectrum split into \(2l + 1\) lines of equal intensity and the magnitude of the splitting between the lines is called the hyperfine coupling constant, denoted \(A\), figure A.3. Thus, for equivalent nuclei, the EPR spectrum will consist of \(2nl + 1\) lines with relative intensities given by binomial coefficients obtained in the expansion of \((1 + x)^n\).

Anisotropic \(A\)-values are characterised by three principal values; \(A_{xx}\) (or \(A_x\)), \(A_{yy}\) (or \(A_y\)) and \(A_{zz}\) (or \(A_z\)). In the case of axial symmetry \(A_x = A_z\) and \(A_x + A_y = A_L\). Shown in figure A.4 is the idealised absorption spectrum and its first derivative for a system of randomly oriented radicals with axial symmetry, anisotropic hyperfine interaction and a single magnetic nucleus of \(I = 1/2\) (for simplicity the g-factor is assumed to be isotropic).

A.2.5 Linewidths

Assuming that the microwave power is sufficiently low (so that no broadening due to saturation occurs) the width of an EPR line can simply be treated as having two contributions. The first is related to the spin-lattice relaxation time (known as \(T_1\)) and characterised by a first order rate constant \(1/T_1\). The second contribution arises because local magnetic fields applied are slightly different at each molecule. For the former, as with NMR, \(T_1\) is a measure of the recovery rate of the spin populations after perturbation. The shorter \(T_1\) the broader the line. For the latter, at any one time, only a fraction of spins is in resonance as the external magnetic field sweeps through a particular 'line'. The observed line is then a superimposition of a large number of individual components, each slightly shifted from the others. The resulting lineshape is Gaussian and this form of broadening is known as 'homogenous broadening'. Homogenous broadening occurs when the average magnetic field is considered to be the same at each dipole, but where the instantaneous magnetic field is not (this gives
Figure A.3. Divergence with field of the $m_s = \pm \frac{1}{2}$ levels in the presence of a single nucleus having $I = \frac{1}{2}$, in the high-field approximation. Note the two allowed transitions involve no change in $m_I$. 
Figure A.4. Idealised absorption (a) and first-derivative (b) EPR spectra for a system of randomly oriented molecules exhibiting g-anisotropy characteristic of (A) axial symmetry ($g_z \neq (g_x = g_y)$) and (B) low symmetry ($g_z \neq g_x \neq g_y$).
rise to a Lorenzian lineshape). For convenience these broadening processes are
classified by a lifetime, $T_2$, such that

$$\frac{1}{T_2} = K\gamma \Gamma \quad (A.12)$$

where $\Gamma$ is half the linewidth at halfheight and $K$ is a constant, and $\gamma$ is the electronic
magnetogyric ratio. For Lorenzian lines $K = 1$ and for Gaussian lines $K = \sqrt{\pi/\ln 2}$.

Since $1/T_1$ is a linear function of lifetime broadening processes a new relaxation time
($T_2'$) can be defined, usually homogenous in nature. $T_2'$ is known as the spin-spin
relaxation time where

$$\frac{1}{T_2'} = \frac{1}{T_2'} + \frac{1}{2T_1} \quad (A.13)$$

For any systems, especially for stable free radicals, $T_1 >> T_2'$, so that, for all practical
purposes $T_2 = T_2'$. 
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Publications
The Site of Electron Capture in Irradiated Deoxynucleic Acid: Cytosine vs. Thymine

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Evidence from e.s.r. spectroscopy for the facile N-protonation of the radical anion of cytosine generated in an aqueous glass, even at 4 K, supports the conclusions that electrons are captured predominantly at thymine when DNA is exposed to ionising radiation.

Electron capture is thought to be a key stage in the radiolysis of DNA.1-3 On the basis of a number of e.s.r. studies it is widely accepted that the major electron gain (and loss) occurs at the heterocyclic bases rather than the sugar or phosphate moieties. Although there is evidence that the electron becomes localised on pyrimidine rather than purine bases, there is considerable controversy regarding the precise sites of trapping, despite the fact that this is a key step in the damage pathway.4 The problem arises because it has been well established by single crystal e.s.r. and ENDOR studies at low temperature using thymine (T) and cytosine (C) derivatives, that the e.s.r. spectra for the electron-capture centres in both cases are characterised by an anisotropic doublet.45 The direction cosines for this show clearly that it is due to coupling to the C-H proton, (1) and (2). These studies failed to show any other coupled nuclei. The result for T" is nicely confirmed by liquid-phase studies which give an isotropic coupling equal to the average of the x, y, and z components for C-H (Table 1), all other hyperfine couplings being small.6

Unfortunately, attempts to prepare C" anions using similar methods have so far failed.4

The spectra for cytosine and thymine derivatives in aqueous glasses rather than crystals are also reported to be closely

![Diagram](Image)
Table 1. E.s.r. hyperfine parameters for thymine (T") and cytosine (C") radical anions and for a protonated form of the cytosine radical anion (CH').

<table>
<thead>
<tr>
<th>Source Medium</th>
<th>Source Medium</th>
<th>1H hyperfine coupling G&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Thymidine*</td>
<td>Single crystal</td>
<td>4</td>
</tr>
<tr>
<td>Cytidine 3'-monophosphate*</td>
<td>Single crystal</td>
<td>4</td>
</tr>
<tr>
<td>Thymine</td>
<td>H2O</td>
<td>278</td>
</tr>
<tr>
<td>Thymine</td>
<td>10 w LiCH2O</td>
<td>77</td>
</tr>
<tr>
<td>Cytidine</td>
<td>10 w LiCH2O</td>
<td>77</td>
</tr>
<tr>
<td>Cytidine</td>
<td>10 w LiCD2O</td>
<td>77</td>
</tr>
</tbody>
</table>

similar. Since most of the studies on DNA are conducted in aqueous media because of the relevance to the situation in vivo, the results from the aqueous glasses may be more comparable with the situation in DNA itself. Using aqueous D2O glasses, which normally give better resolution than H2O glasses, well defined doublets are indeed obtained for both C and T derivatives (Figure 1). These glasses are specially selected because it has been well established that electron-capture by suitable solutes is far more efficient than electron-loss, so there is no ambiguity in assignment of the doublets to electron-capture centres.

For frozen aqueous DNA, one component of the low temperature e.s.r. spectra is also a doublet, almost indistinguishable from those described above. The nature of the controversy concerns whether the radical anions formed in DNA and detected by low temperature e.s.r. spectroscopy are primarily T" or whether C" makes a significant or even major contribution. Given that the e.s.r. spectra for these two species are so similar there is clearly a problem of assignment.

In pursuing this important point we have returned to studies on model mononucleosides and nucleotides in water and aqueous glasses. The latter systems have been shown to be particularly good for forming electron-capture centres. We have confirmed that in all systems T gives a well defined doublet closely similar to the DNA doublet. However, although C gives an apparently identical doublet in D2O glasses, it gives a well defined triplet when H2O glasses are used. The radical anion formed from C in the crystal is a doublet. The extra splitting that arises in H2O glasses is clearly due to a proton derived from the solvent, which means that the species in aqueous glasses is not C" but CCH'. The C-H coupling in CH' is almost identical to C" (Table 1), which rules out protonation on carbon as occurs with T" (3). The most reasonable proposal is that the NH2 group has become protonated rather than the ring nitrogen. Provided rotation of the resulting -NH3+ group is prevented by hydrogen bonds, one large coupling could result, (4).

In these model studies the protonation of C" in aqueous glasses is an extremely facile process, and occurs extensively even at 4 K. Given that protonation of T" occurs in DNA to give TH' has been unambiguously demonstrated, we believe that the protonation of C"; if formed in DNA, might reasonably be expected to occur readily. On the basis of these present results we would expect therefore to detect a triplet in frozen aqueous DNA systems in H2O if there was a significant C" population initially. However, we detect only the doublet, which is replaced by the characteristic eight-line spectrum of TH' on annealing. It is possible that there may be special reasons why protonation of C" might be prevented in duplex DNA but given the ease of this process for C and the behaviour of T" outlined above this seems unlikely.

Figure 1. First derivative X-band e.s.r. spectra for (a) a solution of thymidine in H2O/LiCl glass after exposure to 60Co y-rays at 77 K, showing the doublet assigned to T"; (b) a solution of cytidine in H2O/LiCl glass showing the triplet assigned to CH'; and (c) as in (b) using D2O, showing the doublet previously assigned to C".
Finally, we call attention to a much cited study in which equal concentrations of T and C in water were frozen to 77 K, irradiated, and annealed so that any T" formed would be converted to TH". We have repeated these studies and find that T" and CH" are formed in about equal yields at 77 K, the former being fully converted into TH" on annealing. In the light of our present observations it is clear that this does not mean that C and T have equal electron affinities. Given that the initial electron capture is not selective, as expected, then it is subsequent electron-transfer such as in equation (1) that would establish which centre had the higher electron affinity. Provided that conversion into CH" is fast, then reaction (1) will be blocked, even if the equilibrium of this reaction lies far to the right.

\[
C^- + T \rightarrow C + T\overset{\cdot}{-}
\]  

(1)

We thank the Cancer Research Campaign and the MOD for grants (Leicester).

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Effects of Ionising Radiation on Deoxyribonucleic Acid
Part VI.—Effects of Hydroxyl Radical Scavengers on Radiation Damage to DNA
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Exposure of dilute aqueous DNA to ionizing radiation at ambient temperatures results in indirect damage to the DNA, major reactions being the addition of 'OH radicals to DNA bases and abstraction of C—H hydrogen atoms from deoxyribose units. In order to concentrate on direct damage processes, we have studied frozen aqueous solutions, but it remains possible that some damage is still caused by 'OH radicals generated close to DNA molecules.

In order to check this, we have used two 'OH radical scavengers, dimethyl sulphoxide (DMSO) and t-butyl alcohol (TBA). For both, in low concentrations (<1:1 base-pair), the damage is confined to DNA and exactly corresponds to normal damage in the absence of scavengers. In a second series of experiments, solid solutions containing hydrogen peroxide were photolysed with UV light at 77 K. This gave a poorly defined sextet assigned to sugar radicals or possibly to thymine 'OH-radical adducts, which was not detected in the absence of hydrogen peroxide, or during γ-irradiolysis. We conclude that hydroxyl radical attack is not important for fully hydrated frozen DNA.

The nature and significance of a range of other radicals detected in DNA solutions more concentrated in DMSO and TBA are also discussed, together with the identification of a species giving a broad singlet in the hydrogen peroxide studies.

It is commonly thought that cell death on exposure to ionizing radiation arises primarily as a result of DNA damage. It is therefore important to try to understand the mechanism of radiation damage to DNA. There are clearly two limiting overall mechanisms for primary damage, one being direct interaction between radiation quanta and DNA, the other being indirect damage via attack by water radicals generated by the radiation. In the latter case, damage by hydroxyl ('OH) radicals is thought to be of major significance. Because most studies have been with dilute aqueous solutions in the liquid state, the latter has been far more widely studied than the former. It was originally supposed that 'OH attack was predominantly on the deoxyribose units, especially since this leads to a nice explanation for strand-breaks (SB), but it has been shown recently that, at least with model compounds, addition of 'OH to bases constitutes the major part of 'OH radical attack. It is suggested that this may be followed by hydrogen-atom transfer from a neighbouring sugar unit, as we postulated originally as part of the direct damage mechanism. We stress that our justification for such reactions, which are probably endothermic, was that the C—H hydrogen atoms are 'poised' close to the reaction centre, and that after transfer [reaction (1)] the reactive sugar radicals can react further, thus forcing the equilibrium to completion [reaction (2)]:

\[
\text{base}^\cdot + \cdot \text{C—H} \underset{\text{slow}}{\rightleftharpoons} \text{base-H} + \text{C}^\cdot \quad (1)
\]

\[
\text{C}^\cdot \quad \overset{\text{fast}}{\rightarrow} \text{break-down products.} \quad (2)
\]

It is unlikely that the direct mechanism is of much importance for fluid aqueous DNA, but it may be of considerable importance for DNA in chromatin for two reasons: (i) this is so highly organised and tightly packed that there is not much room for water so that water damage becomes much less important; (ii) nuclear water comprises concentrated solutions of a wide range of organic compounds all of which are reactive towards water radicals. Hence the only radicals likely to attack DNA are those generated close to DNA. This greatly limits the effective aqueous target volume, thereby again reducing the probability of indirect damage. Therefore, direct damage needs to be considered. Our approach, following that of Gregoli and co-workers, has been to use frozen aqueous solutions of DNA. Others have used either dry DNA or partially hydrated DNA, and in particular, Grasslund, Hütttermann and their co-workers have used stretched DNA ribbon, which is largely oriented along the long axis, in order to obtain better EPR information than can be obtained from the frozen solutions.

The frozen solutions that we have used have the advantage that the DNA is fully solvated. Also, it is easy to incorporate additives designed to modify the course of DNA damage. On
freezing, pure ice crystals grow out until a glassy DNA phase solidifies. Additives are totally rejected by the ice, and usually remain in the DNA phase and hence are close to the DNA. This convenient system breaks down if a third phase, comprising pure or hydrated additive, also crystallises out.

Dimethyl sulphoxide (DMSO) is of great importance as an additive to cellular systems, since it acts as a cryoprotector. It is also found to be a radioprotector in that it suppresses radiation-induced cell transformation in vitro.** It is also used extensively in biological radiation studies specifically to scavenge OH radicals.** A disadvantage is the generation of reactive methyl radicals, and, of course, the need for relatively high concentrations if efficient competition is to be achieved.

DMSO reacts slowly with solvated electrons ($k = 1.7 \times 10^9 \text{ mol}^{-1} \text{ s}^{-1}$),* but in the pure solid, reaction with ejected electrons occurs to give methyl radicals, the expected radical-anions being apparently too unstable to be detected.*

$$\text{Me}_2\text{SO} + e^- \rightarrow [\text{Me}_2\text{SO}^-] \rightarrow \text{Me} + \text{MeSO}^+$$

Electron loss gives the radical cation. This species has been prepared unambiguously in CFCl$_3$ at 77 K and has been characterised by a septet EPR spectrum and large $g$-value variation.* In pulse radiolysis studies a transient with $\lambda_{max} = 600 \text{ nm}$ was identified as the parent radical cation, or as a breakdown product therefrom.* In the present work we have found that the parent cation formed in CFCl$_3$ at 77 K has an intense violet colour, indicating an absorption band close to 600 nm. Hence we are able to confirm that the species detected in the liquid state was indeed the parent cation.

t-Butyl alcohol is also favoured as an OH radical scavenger by radiation chemists.** In its reaction with solvated electrons is unimportant, but it reacts very rapidly to give $\text{CH}_3\text{CH}_2\text{OH} + \text{OH}^- \rightarrow \text{CH}_3\text{CH}_2\text{O}^- + \text{H}_2\text{O}$

and the radical cations deprotonate to give $\text{H}_2\text{C} = \text{CHMe}_2\text{OH}$ radicals, or lose $\text{CH}_3$ to give acetone.*

$$\text{CH}_3\text{CHOH} + e^- \rightarrow (\text{CH}_3)\text{CH}_2\text{CO}^- \rightarrow \text{CH}_3 + (\text{CH}_3)\text{CH}_2\text{CO}$$

$$\text{H}_2\text{C}(\text{CH}_3)\text{OH}$$

In our previous study of the action of hydrogen peroxide on DNA,** no significant changes were detected in the absence of radiation, but on exposure to $\gamma$-rays at 77 K the yields of DNA radicals were reduced and HO$_2^-$ radicals, together with species identified as sugar radicals, were detected.* In the present work we have used UV light to generate OH radicals exclusively, thereby avoiding the competing generation of DNA radicals by the direct mechanism. This was one of the first techniques used to generate matrix isolated radicals for EPR study,** and remains a powerful method for studying reactions of OH radicals.

Results and Discussion

DNA + Hydrogen Peroxide

Using light from a high-pressure mercury lamp (mainly 313 + 365 nm) there was no significant DNA damage in the absence of hydrogen peroxide. However, in its presence, spectra in the free-spin region grew in (fig. 2), which at high microwave powers showed a low-field doublet characteristic of HO$_2^-$ radicals.** The central features comprise a broad singlet together with a poorly defined 13 G sextet.

Despite the liquid-phase results, which strongly indicate preferential addition of 'OH radicals to the bases of DNA, our results seem to favour H-atom abstraction from the deoxyribose units for solid systems at low temperatures. Thus, EPR spectra for most possible 'OH radical DNA base adducts have been reported (table 1),** but we have found no evidence for any of these adducts, which are all expected to be stable at 77 K despite their rapid reactions at room temperature. The only reasonable candidate is the thymine 'OH adduct for which we obtained a 20 G quintet in the solid-state (table 1). This differs sufficiently from the 15 G features of fig. 2(a) that we consider that it cannot be a major product.

The sextet may, in part, be due to C$_2$ centred radicals (II). This radical has no $\alpha$-protons, so relatively narrow lines are expected.

$$\text{HO}_2^-$$

Fig. 2. First derivative X-band EPR spectra for a hydrogen peroxide-DNA solution photolysed at 77 K, set at low microwave power, showing features assigned to a sugar radical (a) lines + a central singlet, and (b) at high power, showing features assigned to HO$_2^-$ radicals.
A stick diagram, using the data given in table 1 (included in fig. 2) shows that this assignment is reasonable, but we do not consider that it is fully established. Also, there may be other anisotropic features present from other sugar radicals which are largely concealed by the sextet and singlet. It is, of course, difficult to identify the broad singlet which is always present in the spectra. None of the radicals discussed above is expected to give such a spectrum. There is a contribution from the 'perpendicular' features for HO' radicals [fig. 2(c)] but another centre is also present. Guanine cations (G') give rise to a very similar singlet (same average g value and same width) and we tentatively suggest that these cations may be formed from OH radicals under our conditions. (They are not formed on photolysis in the absence of H2O.)

Absence of high yields of OH radical base-adducts probably reflects the inaccessibility of the bases at 77 K. The most mobile species under these conditions are electrons, which can travel considerable distances along the DNA spine. Protons can also migrate, but OH radicals are likely to be trapped or to react with their near neighbours. There is no clear evidence for trapped OH radicals, but we stress that, in glassy media, their EPR features are not well defined. We suggest that they react with their near neighbours, which are expected to be H2O molecules (giving HO'), and the outer regions of the DNA, which are mainly deoxyribose units. One of the most exposed C—H groups is C1—H, and this may explain why the C1 radical appears to be of importance in this study. The absence of this centre in the radiolysis of

\[ G + \text{OH} = G^+ + \text{OH}^- \]  

At present, we have no other suggestions for the structure of the species responsible for this central feature.
DNA reinforces our conclusion that 'OH radical attack is not of major importance under these conditions.

**DNA–DMSO System**

In our studies of electron-donor and -acceptor additives, there have been clear reductions in the yields of the major DNA damage centres \( \text{C'*} \) and \( \text{T'/C~} \), even at ratios of 1:1. However, for DMSO systems, even at ratios of 1:1 the [G*'] and [T'/C~] are almost unchanged. Although we and others have previously assumed that the major \( \text{e}^- \) capture centre is \( \text{T}' \), there is growing evidence that \( \text{C}'' \) is also important. This result also supports the contention that 'OH radical reactions are not an important source of DNA damage under our conditions.

However, there are interesting effects for higher [DMSO]. Increasing the DMSO: base-pair ratio to ca. 10:1 results in a clear loss of DNA radicals, and the appearance of signals from 'CH\(_3\) and H\(_2\)CSO(CH\(_3\)) radicals (fig. 3). The EPR resembles that obtained from concentrated aqueous DMSO solutions after exposure at 77 K, but the large reduction in [DNA] radicals means that these do not simply arise as a result of damage to a separate H\(_2\)O–DMSO phase. Since the ionization potential of DMSO (9.1 eV) is greater than that of G*’ (8.24 eV) hole transfer should not favour DMSO damage.

The problem is, not the appearance of DMSO radicals, since these must surely be formed at high concentrations of DMSO, but the decrease in DNA radicals, when DMSO is so ineffective at low concentrations [fig. 3(6)]. Probably several factors contribute: (i) the decrease in [H\(_2\)O] in the DNA zone may facilitate electron return thus decreasing [G*’] and [T'/C~]; (ii) the reaction of \( \text{H}^* \text{O}^* \) to give \( \text{G}'' \) may become less favourable than reaction with DMSO to give DMSO*’; (iii) DMSO molecules may intercept electrons before they reach \( \text{T} \) or \( \text{C} \) or before these units have time to relax to form protonated units. Also, electron-transfer between DMSO molecules may help to remove \( \text{e}^- \) from the vicinity of DNA.

**DNA–β-Butyl Alcohol**

As with DMSO, at relative concentrations up to ca. 1:1 (TBA : base-pairs) there is almost no effect on the concentration of DNA radicals or their behaviour on annealing. Despite the relatively high concentration of TBA, there is no detectable yield of TBA radicals. Even 'CH\(_3\) and (CH\(_3\))\(_{2}\)C, which can be detected in very low concentrations because of their narrow features even at 77 K,\(^{13}\) were not detectable. This result accords with those from the DMSO and hydrogen peroxide studies, showing that, in marked contrast to the liquid-phase, attack by 'OH radicals formed by \( \gamma \)-radiolysis, must be insignificant.

Results at higher [TBA] contrast significantly with those for DMSO. Yields of TBA radicals ['CH\(_3\), (CH\(_3\))\(_2\)C and H\(_2\)COCH\(_3\)] remain very low, but yields of DNA radicals [G*’, T'/C~ and 'TH] increase, rather than decrease (fig. 4). Thus TBA appears to act as a radiosensitiser, whereas DMSO acts as a poor radioprotector.

This curious contrast can be understood in the following way. In both cases, the aquted cosolvent molecules are forced into the vicinity of the DNA, thereby potentially increasing the DNA target volume. (This volume effect is well defined for inert salt systems, and will be elaborated separately.) In the case of TBA, electron-capture [eqn (4)] and hole-capture [eqn (5)] are clearly very inefficient, so that both electrons and holes are mobile and can reach the DNA, thereby increasing the yields of DNA radicals.

However, for DMSO, the rates of both \( \text{e}^- \) and hole capture are greater and can compete even with capture by DNA so that DMSO radicals dominate at high concentrations.

**Strand-breaking Studies**

In most of our previous work we have correlated the EPR data with measurements of strand breaks after annealing to room temperature.\(^{10-11}\) In the present work, only TBA systems have so far been studied.\(^{14}\) We note that, in this case, TBA in the low concentration range has very little effect on the number of strand-breaks, in accord with the EPR results, whilst in fluid solution marked protection was observed.

We thank the Cancer Research Campaign (CRC), Ministry of Defence (MOD) and Association for International Cancer Research (AICR).
Fig. 4. (a) First derivative X-band EPR spectrum for a TBA-DNA (20:1) solution after exposure to 55Co-rays at 77 K and annealing to ca. 208 K, showing features primarily assigned to TH radicals. The intensity of these features is ca. twice that in the absence of TBA.

(b) Effect of [TBA] on the intensities of DNA radical signals (G'• + T*/C') at 150 K (-----) and of TH radical signals at 170 K (-----).

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Factors controlling the Site of Protonation of the One-electron Adduct of Cytosine and its Derivatives

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Exposure of cytosine and derivatives thereof to $^{60}$Co $\gamma$-rays at 77 K in a range of aqueous solutions (H$_2$O or D$_2$O) at 77 K gave EPR spectra assigned to the corresponding radical anions. These spectra comprise either doublet or triplet hyperfine features due to one or two protons. Triplet features observed using H$_2$O media become doublets using D$_2$O media. Well-defined triplet spectra were observed using lithium chloride glasses and methanol-water glasses. Using frozen aqueous solutions which give phase-separated solids, spectra ranging from doublets via mixtures to triplets were obtained, depending on the derivative used. It is suggested that the first-formed radical anions are rapidly protonated either on the ring nitrogen [N(3)] or on the amino nitrogen. In the former, there is no extra splitting from the added proton and a major doublet is observed because of hyperfine coupling to the C(6) proton [C(6)—H]. In the latter case, the proton is thought to add along a path normal to the plane of the ring so that overlap with the SOMO is maximized, and there is a large hyperfine coupling. Coupling to the other two amino protons is too small to resolve under these conditions. The fact that the $\sim$N(CH$_2$)$_2$ derivative gives a doublet rather than a triplet supports the postulate that the $\sim$NH$_2$ group is indeed protonated in the triplet species. For the aqueous-methanol glasses, on annealing, this extra doublet splitting is lost irreversibly, probably because rotation of the $\sim$NH$_2$ group sets in, making the three protons equivalent.

As has been shown by Bernhard and co-workers, certain oligomers which give C$^-$ on irradiation give rise to doublets only in H$_2$O glasses. Hence N(3)-protonation is favoured in polymeric systems. Reasons for these differences, and the importance of these results in relation to DNA are discussed.

When DNA is exposed to ionizing radiation at low temperatures two major centres are formed, as judged by EPR spectroscopy. One, thought to be the electron-loss centre, C$^-$, is not considered herein. The other centre, thought to be the electron-gain species, gives a poorly resolved anisotropic doublet of ca. 16 G splitting. For many years this was thought to be due to T$^-$ anions, mainly because thymines itself gives such a doublet on irradiation$^{1,2}$ and because, on annealing, the doublet is partially converted into an octet which is undoubtedly due to protonated T$^-$ ions, where the proton is added to the C(6) ring carbon atom (I). However, Bernhard has long contended that there are serious flaws to this argument, one being that the species identified as T$^-$ is oriented DNA fibres had EPR parameters which were not as close to those obtained from single crystals as might be expected.$^{1,3}$ A major factor in support of this is that relatively dry DNA gives very low yields of TH$^+$ on exposure to ionizing radiation.$^6$

The only reasonable alternative to T$^-$ is C$^-$. This gives a doublet powder EPR spectrum which, at X-band frequencies, is almost indistinguishable from that for T$^-$. (One or both of these anions is expected to be protonated under normal conditions but, for convenience, we use the parent radical anions for purposes of nomenclature). However, in irradiated single-crystals, the doublet EPR spectra for T$^-$ and C$^-$ are clearly differentiated. Also, if ENDOR spectra could be obtained, identification should be easy. Unfortunately, we, and others$^4$ have so far been unable to obtain ENDOR spectra.

Recently, both Bernhard et al.$^5$ and Hüttermann et al.$^6$ have obtained strong evidence in favour of electron capture by cytosine bases, although the latter are reluctant to make a firm identification.$^7$ The former base their conclusions on extremely careful line-fitting procedures using Q-band spectroscopy, which enhances the differences between the two doublets. They then go on to show that irradiation of the tetramer d(CpGpCpT) and a 1:1 ratio of the complementary hexamers d(TpApGpCpGpT) and d(ApGpCpTpGpC) in 12 mol dm$^{-3}$ LiCl glasses gives mainly C$^-$ rather than T$^-$.$^8$ Despite the factors that limit this type of analysis, such as the presence of small amounts of singlet species (e.g. A$^-$ and G$^-$) and changes in parameters induced by solvent relaxation etc., this study is, in our view, qualitatively convincing. Even more so is the new work on oriented DNA$^9$ By incorporating deuterated thymines into the DNA, they show that the spectrum of the major product previously thought to be due to T$^-$ remains unchanged. The EPR parameters actually fit well with single crystal data for C$^-$.$^7$ Thus both groups consider the C$^-$ is the major initial electron-gain centre.

At about the same time the issue was further complicated by our discovery that the radical anion formed by cytidine, a monomer cytosine derivative, in an aqueous LiCl(H$_2$O) medium actually gives rise to a triplet EPR spectrum.$^9$ The triplet changes to the normal doublet when H$_2$O is replaced by D$_2$O. In view of the extensive work carried out on the parent bases of DNA, including cytosine and its derivatives,$^{10-11}$ it is difficult to understand why this triplet had not been observed previously. It seems probable that many workers automatically used D$_2$O or deuterated crystals in order to reduce the linewidths.
Although this result may not be of major importance in relation to DNA itself, it raises a number of interesting questions regarding the sites of protonation. Our aim is to attempt to answer these and related questions.

**Experimental**

Cytosine, 1-methylcytosine, 3-methylcytosine, 5-methylcytosine, cytidine, 2-deoxycytidine (2'5'-CMP), 2-deoxyadenosine-5'-monophosphate (2'-dCP), cytidylyl(3'—5')cytidine (CpC), cytidylyl(3'—5')guanosine (CpG), poly(dG-dC) and poly(dG-dC) were obtained from Sigma Chemical Co. High-purity $\text{H}_2\text{O}$, $\text{D}_2\text{O}$ and $\text{CD}_2\text{OD}$ were obtained from Aldrich Chemical Co. and $\text{D}_2\text{O}$ from Goss Scientific Instruments Ltd. These materials were used without further purification. The $\text{H}_2\text{O}$ was purified using a Millipore 'Multi-Q' system.

**Procedure**

Stock solutions were prepared of aqueous 10 mol dm$^{-3}$ lithium chloride ($\text{H}_2\text{O}$ and $\text{D}_2\text{O}$), and methanol-water ([$\text{CH}_2\text{OH}-\text{H}_2\text{O}$ and $\text{CD}_2\text{OD}-\text{D}_2\text{O}$]) at a ratio of 9:2 (v/v). The aqueous lithium chloride and methanol glasses were made using substrate concentrations between 50 and 100 mmol dm$^{-3}$. Beads were formed by allowing small droplets to fall into a reservoir of liquid nitrogen. Frozen aqueous samples were pretreated by cooling, in liquid nitrogen, a Pyrex tube containing ca. 0.1 ml of a given solution producing solid cylinders (pellets); for most substrates a concentration of 500 mmol dm$^{-3}$ was used; however, for cytosine and 3-methylcytosine only 35 and 60 mmol dm$^{-3}$, respectively, could be accommodated owing to their low solubility.

Samples were irradiated either at 77 K using a Vickers $^{60}\text{Co}$ y-ray source or at 4 K with an X-ray source; irradiations being carried out to a dose of 1 Mrad for the aqueous glasses and 3 Mrad for the frozen aqueous systems. EPR spectra were recorded at 77 K on a Varian E-109 X-band spectrometer, interfaced with an Archimedes computer. The relevant EPR spectra for electron adducts were obtained by (i) warming the frozen aqueous pellets to 135 K to remove masking OH radicals, (ii) for aqueous methanolic glasses, warming to ca. 130 K, allowing the trapped electron to become mobile and removing masking solvent features (such as the $\text{CH}_2\text{OH}$ radical), Fig. 2(a). In aqueous lithium chloride glasses the EPR spectra were obtained directly at 77 K.

As a test to see if electron-adduct radicals were in fact formed in the lithium chloride glass, a powerful electron scavenger ([$\text{K}_2\text{Fe(CN)}_6$]) was employed. Complete suppression of radical anion formation was obtained using 8 mg cm$^{-2}$ ferricyanide in cytidine-10 mol dm$^{-3}$ LiCl.

Samples were annealed either by decanting the coolant from the insert Dewar and recolling when significant spectral changes were detected or by the use of a copper-block cryostat. In the latter the samples were allowed to warm to a predetermined temperature which was maintained for 7 min before recooling to 77 K.

**Results and Discussion**

**Aqueous Lithium Chloride and Methanolic Systems**

Table 1 Results for electron addition to cytosine and a range of its derivatives

<table>
<thead>
<tr>
<th>compound</th>
<th>$\text{H}_2\text{O}$</th>
<th>$\text{D}_2\text{O}$</th>
<th>$\text{LiCl}$*</th>
<th>methanol*</th>
<th>frozen aqueous*</th>
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<td>25.4 (14.1)</td>
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<tr>
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<td>26.7 (15.0)</td>
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<tr>
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<td>35.2 (26.0)</td>
<td>25.6 (14.2)</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
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<td>25.2 (14.0)</td>
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<tr>
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<td>26.6</td>
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<td>33.0 (24.0)</td>
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<td>25.2 (14.1)</td>
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</tr>
<tr>
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<td>—</td>
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<tr>
<td>cytidylyl(3'—5')guanosine [CpG]</td>
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<td>—</td>
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<td>25.0 (13.9)</td>
<td>25.0 (13.9)</td>
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</tr>
<tr>
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<td>—</td>
<td>—</td>
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<tr>
<td>cytidine (at 4 K)</td>
<td>31.4*</td>
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* Two sets of values are used. The main value is a measure of peak-to-peak distances of the outer lines for the triplets ($\gamma$, Fig. 1(a)) and doublets ($\nu$, Fig. 1(d)). The value in parentheses is a measure of total isotropic hyperfine splitting ($\alpha + \nu$) for the triplets ($\gamma$, Fig. 1(a)) and of actual isotropic hyperfine splitting for the doublets ($\nu$, Fig. 1(d)). * Mixture of doublet + triplet.
Fig. 1. First-derivative X-band EPR spectra for 2'-deoxycytidine (50 mmol dm⁻³) in aqueous lithium chloride glasses after exposure to ⁶⁷Co γ-rays at 77 K, showing features assigned to the protonated radical anions; (a) in 10 mol dm⁻³ LiCl-H₂O, recorded at 77 K; (b) warmed to 155 K, recorded at 77 K; (c) simulated EPR spectrum of (a) in 10 mol dm⁻³ LiCl-D₂O, warmed to 155 K and recorded at 77 K; (d) simulated EPR spectrum of (a).

Almost certainly the reason for the dichotomy of results is that the anion can protonate at two different sites, N(3) in the ring, and —NH₂ group (II and III). The N₃—H proton (II) is not expected to give rise to any large extra splitting.

Hence, we should observe only the major doublet from the C(6) proton (other hyperfine splittings are too small to be resolved under our conditions). This is the expected mode of anion protonation. Protonation at the —NH₂ group, to give the —NH₃⁺ "distonic" radical (III) might have been expected to give a 1:3:3:1 quartet from these protons. However, if rotation is seriously restricted, the incoming proton could well approach from 'above' the ring as indicated in (IV). This may, in a protic glass, remain locked by hydrogen bonding. If we assume (arbitrarily) that the conformation is exact (θ = 0°), then given a coupling of ca. 12 G for the added
proton and accepting the simple cosine law, the coupling to the other two protons should be ca. 3 G. This triplet splitting is lost within the linewidth of our spectra. On deuteration to give $-\text{ND}_2$, the 12 G doublet should change to a ca. 2 G triple; this splitting also being lost in the linewidth of the residual doublet.

In this study various experiments have been carried out to check and reinforce our conclusion that the triplet species is really the amino group protonated radical anion ($-\text{NH}^-$). These are (i) for aqueous LiCl glasses, incorporation of a powerful scavenger [$\text{Fe(CN)}_6^{3-}$] completely suppresses the formation of the triplet centre, proving that it must be formed by electron capture. (ii) N(3) methylation has no effect on the results; the triplet spectrum being almost identical (Fig. 2(c)). Thus, it cannot be a proton at N(3) that causes the extra 12 G splitting. On the other hand, the dimethylamino ($-\text{NMe}_2$) derivative fails to produce the extra splitting (Fig. 2(c)) which strongly suggests that protonation of the amino group is responsible. (iii) The results for the $d_5$, $d_6$ derivative show conclusively that the normal triplet spectrum becomes a doublet, thus confirming that the source of the 'extra' splitting is really due to one added proton. We consider that these experiments confirm our original postulate and this is accepted herein.

Typical EPR spectra for a cytosine derivative in aqueous methanolic glasses (CH$_3$OH-H$_2$O and CD$_3$OD-D$_2$O), warmed to ca. 130 K, are shown in Fig. 3(a) and (b). The usual triplets and doublets are obtained in these systems (Table 1).

If structure III is correct, we would expect that rotation or extensive libration would eventually set in on annealing. There is a small increase in hyperfine splitting on warming for most glasses. However, for the aqueous methanol system, at temperatures close to the glass-softening point, the triplet collapses to a very poorly resolved doublet* (Fig 3(c)). We have simulated this using data for the deuterated species to give the normal doublet, with $\text{tm} = 1:3:3:1$ quartet. Thus, it cannot be a proton at N(3) that causes the extra 12 G splitting. On deuteration to give $-\text{ND}_2$, the 12 G doublet should change to a ca. 2 G triple; this splitting also being lost in the linewidth of the residual doublet.

Frozen Aqueous Systems

The reason for concentrating on aqueous LiCl and methanol solutions was to ensure isolation of the substrates and to confine the primary reaction to electron addition. It has been well established that this is the major reaction for dilute substances in such solvents. In contrast, for pure aqueous solutions, phase separation occurs, giving ice crystals and confining the primary reaction to electron addition. It has been seen that base-stacking can be important in frozen aqueous systems, and this may modify the results. Under these conditions, several other species were detected: these will be discussed elsewhere.

DNA Oligomers

Using dGpGpCpGpC Bernhard has established that in LiCl-H$_2$O glasses, C$^-$ forms a doublet species not a triplet species in duplex systems. We have confirmed this, using poly(dG-dC), poly(dG-dC) in a LiCl-H$_2$O glass, Fig. 3(a).

Choice of Protonation Site

The most important aspect of this work is that the issues governing the preferred site of protonation of C$^-$ anions are quite subtle. As Steenken et al. have established, C$^-$ is a strong base, and is rapidly protonated in fluid aqueous solutions. Hence, protonation is expected in protic media, and it seems probable that the parent radical anion has never been detected by EPR spectroscopy. We have tried various aprotic solvent systems using methylated derivatives, but have had no success in generating the parent anions.

Our results suggest that in most neutral glassy systems, the predominant product has the added proton on the $-\text{NH}_2$ group. However, there is a complete switch for the oligomer. Steenken et al. have stressed that in systems containing G-C base pairs (V): the central hydrogen bond is strong and
Fig. 4 First-derivative X-band EPR spectra for cytidine-5'-monophosphate and polycytidylic acid (50 mg cm⁻²) in frozen aqueous solution after exposure to Co γ-rays at 77 K and annealing to 130 K, showing features assigned to the protonated radical anions; (a) cytidine 5'-monophosphate-H₂O; (b) in D₂O; (c) poly C -H₂O; (d) in D₂O, short, and neutralisation of C~ by the proton in this bond should be strongly favoured. Clearly this will occur preferentially in any duplex system.

Our results confirm that protonation of the —NH₂ group is highly competitive with N(3) protonation. It seems to be favoured for monomeric cytosine derivatives, whereas N(3) protonation is strongly favoured for C~ in duplex DNA. This switch can readily be understood in terms of the duplex structure. However, the —NH₂ group is not predominantly protonated for poly C (Fig. 4(c)), so the duplex structure is not a complete requirement. It is not clear to us why this change should occur on going from monomer to polymer.

The results obtained on exposure of cytidine in a LiCl-H₂O glass to X-rays at ca. 4 K suggest that protonation at —NH₂ is still extensive, though there seems to be some admixture of a doublet centre. This strongly supports our contention that the —NH₂ "lone pair" is already hydrogen bonded in these solutions prior to electron addition, so that H⁺ transfer (by tunneling) remains facile even at 4 K.

For frozen aqueous systems the results are more subtle. With cytosine itself there seems to be mainly N(3) protonation, whereas for CMP, dCMP, 2'-dC and cytidine, protonation at both sites occurs. In the former, base stacking is extensive in the frozen phase-separated systems, but this is less likely to occur for the remainder. We stress that for poly C, taking into account radicals formed as a result of electron-loss (e.g. β in Fig. 4(c)), mainly a doublet is obtained. Despite poly C giving mainly a doublet EPR spectrum for C~, the dimers C₃C and C₃G in LiCl-H₂O give the triplet predominantly, which becomes the usual doublet in LiCl-D₂O glasses (Table 1).

It has been suggested that a major factor contributing to this preference (III) is coordination of Li⁺ (in the LiCl glasses) to N(3). Such coordination would certainly prevent protonation at this site. However, we would not expect a preponderance of direct bonding in this aqueous medium. Infrared results obtained from frozen methanolic and aqueous electrolyte solutions showed that solvent shared ion-pairs represent the major form of interaction, so units such as VI are predicted. These, of course, should be good proton donors. Furthermore, our results for aqueous methanolic systems, which give good triplets (Fig. 2(b)), show that this theory cannot explain the preference for —NH₂ protonation.

Finally, we stress that although the doublet spectra obtained for monomeric cytosine derivatives in LiCl-D₂O or other deuterated glasses look similar to the doublet for C~ in DNA polymers, they must differ in their EPR parameters because they are due to quite different species, the —ND₂⁺ derivative and the —N(3)—D⁺ derivative.

Protonation on Carbonyl Oxygen

With G-C pairs in duplex DNA both N(3) and the carbonyl oxygen of C act as hydrogen bond acceptor sites, whereas the —NH₂ group acts only as a proton donor. Thus it might be argued that oxygen should be the alternative site for protonation rather than the —NH₂ group. It is unlikely that such protonation would give rise to significant hyperfine coupling from the added proton, since this is expected to remain in the plane of the radical, and the expected spin density on oxygen is low. Carbonyl groups generally form two hydrogen bonds in water, but the favoured sites are in-plane, as is the case for H bonding to C=O groups in duplex DNA. However, Sagstuen et al. have recently shown that when T~ is formed at 4 K in crystals of thymidine an extra doublet is found which is lost using crystals grown from D₂O. This certainly occupies the out-of-plane site (VII), but this arises because of the specific crystal structure for thymidine. In their pioneering studies of DNA base crystals, Box and Budzinski missed this proton splitting because their crystals were always grown from D₂O. One result that strongly suggests —NH₂ protonation rather than C=O protonation in the present work is that for the —N(CH₃)₂ derivative. This gave only a doublet under all conditions. Thus this modification has caused a
We see no reason for such a switch if the remote C=O group were involved. In contrast, the switch is quite reasonable for amine protonation. One factor is that in order to become 'tetrahedral' the two bulky methyl groups have to move considerably. The other, probably more important factor, is that of hydrogen-bond cooperativity. If, as expected, the two N—H protons are acting as hydrogen bond donors in these solutions, this will greatly enhance the basicity of the nitrogen ' lone pair'. This effect is obviously absent in the —NHCH₂₂₃ derivative. We conclude that protonation of the —NH₂ group is favoured over that of the >C=O group.

Switch in the site of protonation [almost certainly to N(3)]. We see no reason for such a switch if the remote C=O group were involved. In contrast, the switch is quite reasonable for amine protonation. One factor is that in order to become 'tetrahedral' the two bulky methyl groups have to move considerably. The other, probably more important factor, is that of hydrogen-bond cooperativity. If, as expected, the two N—H protons are acting as hydrogen bond donors in these solutions, this will greatly enhance the basicity of the nitrogen ' lone pair'. This effect is obviously absent in the —NHCH₂₂₃ derivative. We conclude that protonation of the —NH₂ group is favoured over that of the >C=O group.

Significance for Radiation Effects in DNA

Unfortunately, for DNA protonation of the —NH₂ group is not expected, and hence the present results are of no assistance in attempts to solve the problem of the site(s) of electron capture when DNA is irradiated. The crucial issue in the competition between capture at C or T is the rate of relaxation leading to a deep trap. This is expected to be extremely fast for C — since the 'central' proton, which is always present, only has to move ca. 1.0 Å, and is ideally placed for transfer. Very little structural change is required and changes in solvation should be small. It is generally supposed that T" does not protonate initially, but protonates irreversibly on C(6) as the temperature is raised. If that is correct, it may seem surprising that any T" is formed. One possibility is that all the initial electron capture gives C" [—C(OH)₂].
Effects of Ionizing Radiation on Deoxyribonucleic Acid. Part 7. Electron Capture at Cytosine and Thymine

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Exposure of a range of DNA samples in various media to ~7Co γ-rays at 77 K gives electron-capture centres characterized by an EPR doublet with A(H) of ca. 16 G. Since the electron-adducts of C and T give very similar doublet EPR spectra in irradiated DNA, it is difficult to judge the proportions of C* and T* formation by inspection. The possibility, suggested by others, that computer fits can be used to give a quantitative measure of these species is discussed. However, in view of the variability of the features directly assignable to C* and T* units in different environments, we suggest that this approach has only qualitative significance.

The alternative method involves annealing to convert T* into TH' radicals in which a hydrogen atom is added to C6, the resulting radical having a completely characteristic octet EPR spectrum. It is argued that the ejected electrons move through the stacked DNA bases, becoming trapped at C or T depending upon the relative rates at which C** and T** are protonated to give C***(H+) and T***(H+), and the factors controlling these rates are discussed. The use of lithium chloride glasses completely suppresses the formation of G** centres, leaving well-defined radical-anion spectra, but on annealing, conversion to TH' is negligible despite the rapid, and complete, loss of the doublet species. This result is discussed in terms of reaction with Cl2 radicals formed in abundance in these glasses.

Studies designed to detect any site-specificity in the DNA damage leading to strand breaks suggest that all possible sites are damaged. These results strongly support the postulate that yields of C* and T* are comparable. The possibility that some A* cations are formed in addition to G** cations is also considered in the light of these results.

In our studies of radiation damage to DNA,1,2 we have largely confined our attention to DNA itself because of the extensive work previously carried out by others using single crystals or frozen solutions of bases, nucleosides, nucleotides and small oligomers.1,11 The system that we selected was frozen aqueous radical-anions. These systems are phase separated on cooling into a pure ice radical-cations. Similarly, one might expect that the electrons trapped into the completely aquated DNA in the liquid state. The only radical detected in the ice phase at 77 K is OH, which has a characteristic EPR spectrum14 and is largely converted into hydrogen peroxide on annealing to ca. 130 K. These radicals are not involved in any reactions of DNA.

When aqueous solutions of DNA are exposed to ionizing radiation at low temperatures, two major signals from DNA radicals are detected by EPR spectroscopy. One, a broad singlet, is generally assigned to some form of electron-loss centre based on guanine, and the other, a doublet, is assigned to centres derived either from cytosine radical-anions or thymine radical-anions.

In earlier EPR studies of frozen irradiated DNA it was generally assumed that the doublet feature is assignable to T* electron-gain centres, and the singlet to G** electron-loss centres, although Bernhard warned that the former assignment could be wrong. We stress that this is a surprising result. Ionizing radiation generally damages indiscriminately. So initial electron-loss centres should include H2O+ (from solvating water), (RO)2PO3−, sugar radical-cations, and all four base radical-cations. Similarly, one might expect that the electrons would react at phosphate groups or with any of the four bases. The results require migration of holes and electrons at rates greater than those for proton or group loss. They also require that trapping in some form is ultimately specific and effective.

This is almost certainly induced by proton gain and loss from (base) and (base) centres.

The T* assignment was given because of conversion of the 'doublet' centre into the completely characterized TH' radical on annealing.15 The warning was given because the 'powder' EPR spectrum for C** was found to be almost indistinguishable...
able from that for T". This warning was reinforced by studies of oriented DNA ribbons. The results were interpreted in terms of G" and T" but Bernhard stressed that the well resolved spectrum assigned to T" gave results which were not in good accord with those for this radical trapped in single crystals.

This issue has now been reopened, largely as a result of renewed attacks on the problem by Bernhard and coworkers. All these groups have come to the conclusion that the major anionic centre formed in DNA at low temperatures is actually C" not T". Our aim has been to endeavour to assess the proportion of T" and C" centres formed in the systems we have normally used. We, and others, generally represent these primary centres as G" and T" in DNA and the polynucleotides (Poly[dG-dC] and Poly[dA-dT]) glasses were prepared by mixing 0.01 mol dm⁻³ of solute with an equal volume of saturated LiCl solution. Cylindrical pellets were made by freezing the solutions in tin foil moulds. Frozen aqueous DNA (50 mg cm⁻³) were prepared by freezing ca. 0.3 cm³ of solution in a Pyrex tube.

Samples were γ-irradiated at 77 K using a Vickers 42Coγ-ray source; irradiations were carried out using doses of ca. 1 Mrad for the glasses and 4 Mrad for the frozen aqueous DNA. UV irradiations were carried out at 77 K using a Hanovia high pressure mercury arc. EPR spectra were recorded at 77 K on a JEOL JES-RE1X X-band spectrometer, interfaced with an Archimedes computer. The radical-anion EPR spectra in aqueous lithium chloride glasses were obtained directly at 77 K. To test whether electron adducts do form, and that no sole radical-cations are formed in the lithium chloride glass, a powerful electron scavenger [K₃Fe(CN)₆] was employed. Complete suppression of radical-anion formation was obtained with 8 mg cm⁻³ ferricyanide with no evidence of solute radical-cations. Samples were annealed using a copper block cryostat. Typically, they were allowed to warm to a predetermined temperature which was maintained for 8 min before recouring to 77 K.

Pellets of the γ-irradiated frozen aqueous DNA samples were annealed to ca. 130 K to remove OH radicals present in the ice phase. Radical growth and decay studies were achieved by stepwise annealing up to 270 K. The EPR spectra of the UV-irradiated frozen aqueous DNA were obtained directly at 77 K and the TH" decay profile was measured upon annealing using the height of the seventh line to measure the relative amount of TH".

**Isolation of EPR Spectra for Individual Primary Centres.**—The primary radical centres under direct damage conditions in DNA are radical-anion (T"/C") and radical-cation (G"*/A") centres giving rise to poorly resolved composite EPR spectra (Fig. 1). The radical-anion doublet (Fig. 1) can be isolated by subtraction of spectra obtained at high and low temperatures which was maintained for 8 min before recouring to 77 K. The first results that we obtained, which indicated that the G" + T" theory was an over-simplification, came from strand-break specificity studies. We found no evidence for preferential breaks in G, T regions under direct damage conditions. This work is described in detail herein.
give similar doublet spectra. However, these are due to the
-ND$_3^+$ species and should not be used as a measure of the
C"(H$^+$)[(N3)-H$^+$] species found in DNA and other duplex
systems, since the two radicals must surely differ.

As is evident from Fig. 2 the task of differentiating between
the T$^-$/T$^-$[H$^+$] and C$^-$/C$^-$[H$^+$] doublets is a formidable one.
Comparisons of DNA$^-$ spectra with computed mixtures of the
C$^-$ and T$^-$ doublets suggest the contribution of C$^-$ to be
at least 90%. A simple and reproducible measure of comparison is
the separation between points of maximum slope ($\Delta$) (Fig. 2),
which is in agreement with the visual comparison. However,
in contrast to this measure, comparison of the depth of the
switchback ($\alpha$,$\beta$) (Fig. 2) does not indicate such a large C$^-$/T$^-$
ratio (ca. 60:40), but owing to the variability of this factor from
tax sample to sample we place more weight on the measure of $\Delta$
values between the two extremes should be expected, however,
this does not occur and the $\Delta$ values remain almost constant
(Table 1).

We stress that non-polymeric cytosine derivatives in
0,0 also
duct to the anionic species spectra of the polymers is negligible.

Electron affinities of the pyrimidines appear to be much greater
than those of the purines, and consequently, spectra from
poly(dA-dT) in 10 mol dm$^{-3}$ LiCl glasses. In these systems, electron-loss
centres are primarily C$^-$/T$^-$ radicals and the radical-anion
centres may themselves
be quite variable for different systems and in different environ­
ments. We suggest that such computational procedures should
not be taken as giving accurate measures of the relative con­
centrations of C$^-$ and T$^-$ centres in DNA. Another reason
for making this suggestion is that there are clearly environ­
mental effects on the EPR parameters. These will be exag­
gerated for C$^-$/T$^-$ centres in DNA rather than in benchmark spectra since there will be a wide range of different
local environments in DNA which will tend to broaden the
spectra and hence make any differentiation even more difficult.
Because of the extreme and apparently random variations in
the centre regions of these spectra (Table 1), computer fits, in
our hands, are also scattered with no significant trends.
Consequently, we feel that no clear distinction between the C$^-$/
and T$^-$ centres can be made.

Electrostatic Competition in Thymine and Cytosine Nucleotides and
dinucleotides.—Our results establish that for irradiated frozen
aqueous DNA, the yield of TH$^+$ radicals is at least 36%, based on
the initial radical-anion yield at 130 K (it is assumed that there is
a 50:50 distribution of radical-anions and radical-cations at this
temperature). Thus either the primary yield of T$^-$ is at least 36%,
or some electron transfer from C$^-$/T$^-$/ TH$^+$ must occur prior to the
formation of TH$^+$. Such transfer is, of course, invoked in the
initial stage of capture of the mobile electrons and, if it were to
occur, it surely represents a slow extension of this process.

If the computer analyses of the initial EPR doublet
assigned to anionic species are accepted, it becomes necessary to
assume a slow, thermally activated step in which C$^-$ ...
Since the added proton leads to an extra hyperfine splitting. If T" was not protonated, electron-transfer would occur to favour C"(H") formation. Since this is not found even on warming to 140 K, we conclude that T" must also be protonated, presumably at the O; carbonyl oxygen.

In contrast, if the T and C bases are held close together, as in TpdC, intramolecular electron-transfer will be very much faster than the intermolecular transfer discussed above and might be expected to compete effectively with protonation (Scheme 2).

![Scheme 2](image-url)

**Scheme 2** Intramolecular electron-transfer and protonation in dinucleotides

Hence, it should be driven by the differences in electron affinities. In this case, computer simulation of the electron addition spectrum of TpdC at 77 K using the individual base spectra indicates the electron distribution to be approximately 37%, T"/(T"/(H")) and 63%, C"/(H") (Fig. 3), which implies that cytosine has the greater electron affinity. It also implies that protonation of T" still competes to some extent with electron-transfer, since transfer to C is by no means complete.

An alternative explanation may be that the C"/T" ratio does not pertain to the relative electron affinities of C and T but rather reflects a more rapid protonation of C". We stress that in TpdC, C" is again protonated in such a manner that it gives a triplet EPR spectrum, making analysis easy and confirming that protonation does still occur.

Qualitatively, these results are significant to the DNA problem. They confirm the importance of electron-transfer and, in particular, of protonation in the process of final electron trapping and that both base-anions become protonated. They also show that cytosine has a higher electron affinity than thymine and/or protonation at C" is faster than at T".

There are major differences compared with the bases in DNA. One is that C" protonates at an alternative site. Another is that C and T bases are no longer necessarily adjacent. Also in DNA relative electron hole migration is extensive prior to trapping.12 Finally, base-stacking in the dinucleotide is unlikely to be as efficient as in DNA. Thus qualitative comparison of TpdC and DNA is of no use. The major conclusion, however, is that in order that electrons be permanently trapped to give T", it almost certainly becomes protonated otherwise migration to C would be essentially complete. We stress that O-protonation of T" occurs readily at 4 K in crystalline thymidine,10 so there is good precedent for this postulate even though it is not favoured by others.16 It has been claimed that O-protonation of T" does not occur in neutral glasses.11,12 This is based upon the fact that line-width broadening of the doublet, presumably due to O-protonation, is observed in acidic glasses. However, this is not a compelling argument since the -O-H coupling is expected to be small and a function of the orientation of the O-H bond relative to the radical plane, falling to zero close to this plane.
course, there may well be other pathways of reaction for the annealing leads to the growth of TH' features (Fig. 7). The major difficulty with this method of assessing \[ \text{TH'} \] is that decay of TH' may set in before formation from C" or T" (H') is complete. Also, of course, there may be other pathways of reaction for the thymine radical anion other than C\" protonation. The extent of concurrent formation and decay of TH' on annealing can be estimated by generating TH' in frozen aqueous DNA at 77 K using ultraviolet light. This gives TH' radicals in good yield, and the thermal decay curve can be monitored (Fig. 8). By matching this with the high temperature region of the rise and decay curve for \( \gamma \)-irradiated TH' systems it is evident that a reasonable estimate of the total yield of TH' can be obtained. In this manner we have calculated that the maximum experimental yield of TH' at ca. 200 K is 18 ± 1\%}. The contribution of T" to the overall anionic doublet must therefore be at least 36 ± 2\%, assuming 30\% radical-anion contribution to the 130 K DNA spectrum.

Thermally-induced Transfer of e\( \sim \) from C\" Units to T in DNA.—In order to reconcile the computer results which require almost complete C" formation at 130 K with our results for TH' which require less than 64\% C" formation, it is necessary to postulate a thermally-induced electron-transfer mechanism from C" to T. If this does occur, it simply introduces a more complicated route to the 64\% value, that we expect to detect some electron return to the cation centres. In the light of our results, the process shown in Scheme 3 is required.

\begin{align*}
\text{Step (1)} & \quad \text{C}(\text{H}^+) + \text{H}^+ \quad \rightarrow \quad \text{C} + \text{H}_2 \\
\text{Step (2)} & \quad \text{C}^- + \text{T} \quad \rightarrow \quad \text{C} + \text{T}^-
\end{align*}

\textbf{Scheme 3} Thermal migration of electrons from C" centres to T

Step (1) requires loss of a proton from C\" (H\*) but since C" has a high proton affinity this stage should have a very low probability. Step (2) is the normal process that occurs in the primary ionization stage. Presumably, it can involve considerable movement of e\( \sim \), or just transfer to an adjacent unit. This reaction is reversible and since protonation of C" is extremely fast \[ \text{step (1)} \] the lifetime of T" will be short. Step (3) must be slow since it cannot proceed via movement within hydrogen bonds, these being of no importance in carbon protonation. Thus it will hardly compete with step \( \text{(-2)} \) and step \( \text{(-1)} \). These considerations make Scheme 3 unlikely. Another aspect of this mechanism is that, if it is the main route to TH', why is it not all of the C" ultimately removed to give TH'? Also, if the electron can enter into thermally-induced transfer, we would expect to detect some electron return to the cation centres. This will result in thermoluminescence which is an extremely sensitive test for electron return. We know of no reports for this in the required temperature range despite very careful studies. In our work we have not been able to pick up any light emission.

Thus, although we cannot rule out Scheme 3, we strongly favour our proposed mechanism which fixes electrons on T in order to alter the computed relative contributions. We find that very small changes are required to move from the SL.17 result of Sevilla et al. to our result of 64-36 based on TH' formation.

**Computer Estimates of the Concentrations of C" and T" Centres.—** When a computer is used to analyse similar spectra, it will generally lead to an apparently clear conclusion. The trouble is that the greater the similarity between the two spectra, the smaller are the changes required, anywhere in the spectrum, to alter the computed relative contributions. We find that very small changes are required to move from the SL.17 result of Sevilla et al. to our result of 64-36 based on TH' formation.
changes that are as large as the differences between the two effects and/or solvent effects on the EPR parameters, and "For C 2. Ha is assigned to the hydrogen atom above the ribosyl plane. Other species that have not been allowed for correctly. (//) site to the hydrogen furthest away.

Our studies suggest that such variables can make spectral changes that are as large as the differences between the two 'benchmark' spectra that are recommended. There is clearly a limit at which such computer analysis of similar spectra must fail. The trouble is that an apparently good answer may still be forthcoming. We conclude that the issue remains an open one but, in view of all the results presented herein, we favour the clear-cut results for TH' formation as an indicator of the extent of T' formation.

TH' Yields in LiCl Glasses.—The maximum yield of the TH' octet after γ-radiolysis of TMP in LiCl glasses is small and the signal is lost at much lower temperatures compared with frozen aqueous solutions. These glasses show intense EPR features for Cl_2^- and probably ClOH^- radicals after irradiation. These features are lost in the same temperature range as those for T" and we suggest that reaction (1) may be responsible. Such a reaction is less probable for DNA LiCl systems since Cl_2^- can react as an electron acceptor, especially with the purines. In fact, we have shown that Cl_2^- is able to cause one-electron oxidation of DNA. Nonetheless, in view of a similar rapid loss of DNA' on annealing, it seems that reaction (2) may still be important. In any case, the very low yields of TH' (5-10%) cannot be taken as evidence for or against an increase in the C'"/T'" ratio with added salt.

Factors Affecting the Relative Yields of T" and C".—Using the TH' yield criterion, our results show that as the concentration of water is reduced below the 100% humidity value, the relative yield of T" is reduced, such that, for 'dry' DNA, C" dominates. This accords with the results for oriented DNA ribbon, which are quite definitive in showing that T" is a minor component. These results can most readily be interpreted in terms of a change in the relative protonation rates for C" and T". In duplex DNA, the degree of hydrogen bonding for cytosine, particularly at N(3), within the C-G base pair, is independent of water concentration, whereas the hydration of the C=O group in thymine is expected to decrease as water is removed. We suggest that this is the factor which is responsible for the relative increase in [C"]. As water is removed, so the rate of protonation of T" is reduced, whilst that for C" remains relatively constant. Hence trapping at cytosine becomes progressively more important.

When salts (LiCl and NaCl) are added to aqueous DNA there appears to be a reduction in the relative yield of TH' even at low salt concentrations. The addition of these salts is known to dehydrate the DNA and cause conformational changes. In these cases, we suggest that the C=O groups of thymine become partially dehydrated, so that T" protonation is inhibited thereby altering the extent to which the electrons are captured to give T"-(H^+). centres. The large apparent C"/T" ratio for DNA in 10 mol dm^-3 LiCl glasses may possibly be a result of this phenomenon, in which case these salt solutions would not be applicable as a measure of the electron distribution in the frozen aqueous systems.

We stress that for the protonation of T" we are not postulating the simple reaction (3), since this would probably favour the reverse process. We suggest that a chain of water molecules is required, as in Scheme 4, thereby removing the OH^- ion from the vicinity of the T"-(H^+) unit, with the formation of a remote X^- anion. Any of the more acidic protons in the neighbourhood should suffice. Clearly, loss of water will

<table>
<thead>
<tr>
<th>5'-deoxyribose</th>
<th>Distance/Å</th>
</tr>
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<tbody>
<tr>
<td>C5 to C1-H</td>
<td>3.49</td>
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<tr>
<td>C5 to C2-Ha</td>
<td>3.93</td>
</tr>
<tr>
<td>C5 to C2-Hb</td>
<td>3.29</td>
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<td>C5 to C3-H</td>
<td>5.79</td>
</tr>
<tr>
<td>C5 to C4-H</td>
<td>6.69</td>
</tr>
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<td>C5 to C5-Ha</td>
<td>8.97</td>
</tr>
<tr>
<td>C5 to C5-Hb</td>
<td>7.52</td>
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<table>
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<tr>
<th>N'-deoxyribose</th>
</tr>
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<tbody>
<tr>
<td>C5 to C1-H</td>
</tr>
<tr>
<td>C5 to C2-Ha</td>
</tr>
<tr>
<td>C5 to C2-Hb</td>
</tr>
<tr>
<td>C5 to C3-H</td>
</tr>
<tr>
<td>C5 to C4-H</td>
</tr>
<tr>
<td>C5 to C5-H</td>
</tr>
<tr>
<td>C5 to C5-Hb</td>
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<table>
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<th>3'-deoxyribose</th>
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</thead>
<tbody>
<tr>
<td>C5 to C1-H</td>
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<td>C5 to C3-H</td>
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<tr>
<td>C5 to C4-H</td>
</tr>
<tr>
<td>C5 to C5-H</td>
</tr>
<tr>
<td>C5 to C5-Hb</td>
</tr>
</tbody>
</table>

* For C 2. Ha is assigned to the hydrogen atom above the ribosyl plane. I.e., on the same side as the base residue and the C 5 moiety, and Hb to the hydrogen below the ribosyl plane. For C 5. Ha is assigned to the hydrogen atom closest to the stacked base interior of the DNA, and Hb to the hydrogen furthest away.

Difficulties include (i) the presence of low concentrations of other species that have not been allowed for correctly, (ii) site effects and/or solvent effects on the EPR parameters, and (iii) the translation of spectra obtained from small units to DNA itself. Our studies suggest that such variables can make spectral changes that are as large as the differences between the two 'benchmark' spectra that are recommended. There is clearly a
break the chain and hence inhibit T$^-$H$^-$ formation. For cases
mediated by hydroxyl radicals, this would also be the case.
Furthermore, if both of these radicals are readily detected by EPR spectroscopy* but under
normal circumstances these are not detected. Secondly, we have
previously suggested that under conditions of direct damage
to DNA strand breaks must arise from the initially formed radical-
cations. Furtherm ore, if both of these radicals are
sugar are appropriately poised.
It is difficult to interpret these data quantitatively in terms of the
ratios of the initial base radicals because of the considerable
uncertainties surrounding the rates and efficiencies
with respect to the origin of single and double strand breaks the same arguments apply
even if the initial radical population is more heterogeneous, as
now seems likely.
For base-centred radicals to give rise to strand breaks we have
previously proposed that an intramolecular hydrogen atom abstraction from a neighbouring sugar residue must at
some point take place. Computer modelling has suggested that
C2$^-$H$^-$ and C1$^-$H$^-$ the 5'-deoxyribose residue next to the
base radical is the nearest sugar C-H group for DNA in the B
conformation (Fig 9 and Table 2). Based on this we reason
that sites adjacent to either T or G should suffer greater damage
than with A or C as neighbours. To test this hypothesis
we promote such a pathway by inclusion of H$_2$O$_2$, sugar
atom abstraction does not only occur from the
neighbouring sugar and therefore that high base specificity in
the formation of the radical-cation and -cation would not be
folllowed by subsequent sequence specificity in the development
of strand breaks. We do not feel this is likely since we believe
that a major driving force for the hydrogen atom transfer must
be its facilities for juxtaposition with respect to the base radical.
Inspection of models suggests that the C-H's of the neighbouring
sugar are appropriately poised.
Sites of Strand Cleavage in Irradiated DNA.—We have
previously suggested that under conditions of direct damage to
DNA strand breaks must arise from the initially formed radical-
cations. Furthermore, if both of these radicals are capable of initiating strand breaks we were able to propose a
novel mechanism to account for the surprisingly large numbers
of double strand breaks that arise. Although we discussed our
early results in terms of G$^\prime$ and T$^\prime$, with respect to the origin
of double strand breaks that arise in a largely non-sequence-specific
manner.
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