SPECTROSCOPIC STUDIES CONCERNING
THE GAMMA IRRADIATION OF
DEOXYRIBONUCLEIC ACID

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by

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This thesis is dedicated to Wendy, Mum, Dad and Vicky.
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Spectroscopic Studies Concerning The Gamma Irradiation Of Deoxyribonucleic Acid.

Carl M. Blackburn

Abstract

Infrared spectroscopy has been used to investigate glassy and ice-like water in frozen DNA solutions. Using a solvent comprising HOD in D2O, the O-H stretch band in the infrared is sharp. However, HOD in glassy water regions contributes to a broader feature. The extent of phase separation was assessed by band analysis of these two components. The amount of glassy water was determined and the DNA radiation target was described according to a three zone model.

The above technique was extended to investigate phase separation in frozen aqueous DNA solutions containing LiCl or NaCl. Changes in the mass of the glassy water-DNA-salt phase were correlated with an increase in DNA radical yield detected by ESR spectroscopy. Added salt increased the DNA radiation target and a salt hydrate (NaCl.2H2O) was formed on freezing solutions containing NaCl. Hence, NaCl contributes two moles of water, as well as the mass of the salt, to the DNA radiation target. A salt hydrate was not detected in solutions containing LiCl as LiCl enters the DNA glassy water phase. Glassy water formation depended on the ratio of LiCl to DNA base pairs and not solely on LiCl concentration.

NMR spectroscopy was used to investigate the release of sodium ions from DNA in aqueous solution. When native (double stranded) DNA was irradiated the Na-23 NMR band narrowed. Line narrowing was examined in conjunction with relationships describing dose yields of single and double strand breaks. Line narrowing was sensitive to single strand breaks but not double strand breaks. A single strand break resulted in the net release of approximately eight sodium ions per DNA base pair from native DNA and seventeen sodium ions per nucleotide from single stranded DNA.
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Chapter 1

Introduction
Introduction

This chapter outlines the reasons why DNA is studied as a "radiation target". Particular emphasis is placed on the use of Co-60 because this was the gamma radiation source used in the research reported in this thesis.

1.1 Deoxyribonucleic Acid

Some understanding of the chemical and physical structure of DNA is necessary in order to understand radiation induced DNA damage. A discussion of the biochemical aspects of DNA structure can be found in current biochemistry text books; e.g. reference 1.

1.1.1 Chemical Constituents

Deoxyribonucleic acid is a polymer constructed from nucleotide repeat units. A nucleotide comprises a phosphate group, a deoxyribose sugar and a base. The sugar is called deoxyribose because it has one less hydroxyl group in the C-2' position than a ribose sugar (figure 1.1). By convention, the sugar atoms are labelled with primed numbers as shown in figure 1.1. A nucleotide contains one of four heterocyclic bases, two of which are derivatives of purine and two which are derivatives of pyrimidine. The purines are adenine (A) and guanine (G); the pyrimidines are thymine (T) and cytosine (C), figure 1.2.

In DNA the deoxyribose sugar is substituted at the C-1' position, and is linked to either the N-1 position of the pyrimidines or the N-9 position of the purines. The resulting sugar-base sub-unit is called a nucleoside. Thus the four nucleoside sub-units in DNA are deoxyadenosine, deoxyguanosine, deoxycytidine and deoxycytidine.

Nucleotides are phosphoric acid esters of nucleosides in which the phosphoric acid forms an ester with one of the hydroxyl groups of the sugar. In DNA nucleotides, the phosphate ester occurs at C-3' or C-5' positions on the sugar.
Figure 1.1 $\beta$-D-Ribofuranose and $\beta$-D-2'-Deoxyribofuranose [1]

\[ \text{\textbeta-D-Ribofuranose} \]

\[ \text{\textbeta-D-2'-Deoxyribofuranose} \]

Figure 1.2 Adenine, Guanine, Cytosine and Thymine [1]

\[ \text{Adenine (A)} \]

\[ \text{Guanine (G)} \]

\[ \text{Thymine (T)} \]

\[ \text{Cytosine (C)} \]
Nucleotides that occur in the free form in cells are predominantly those with a phosphate group attached at C-5'. In aqueous systems at pH 7 the two protons attached to the phosphate group are dissociated and the nucleotides are primarily in the form $R-O-PO_3^{2-}$ (aq), where $R$ is the nucleoside group.

A polynucleotide is formed by joining several nucleotides together, the bridging group being a phosphodiester which links to the C-5' atom of the deoxyribose sugar of one nucleotide and to the C-3' position of the deoxyribose sugar in the other nucleotide.

DNA is a duplex of two polynucleotide strands. The sugar phosphate chains form the backbone of DNA and each base is paired with a base on the opposite polynucleotide strand. Under physiological conditions, DNA is present in aqueous solution as DNA$^{2-}$ (aq); i.e. DNA is a polyanion with one negative charge per phosphate group.

1.1.3 DNA Structure

DNA consists of two polynucleotide strands which are wound around each other to form a double helix. These two polynucleotide strands are antiparallel; i.e. their C-3', C-5' internucleotide phosphodiester "bridges" run in opposite directions. The DNA helix is analogous to a spiral staircase; the banisters are the two strands of sugar phosphate esters and the steps are hydrogen bonded bases. The bases are uniquely paired, adenine is always hydrogen bonded to thymine (A:T) and guanine is always hydrogen bonded to cytosine (G:C). A schematic representation of the DNA double helix is shown in figure 1.3 [2].

Watson and Crick [3,4] first proposed the helical structure for DNA in 1953, and their model has been found to be essentially correct. However, DNA can exist in different conformational forms. Each form is distinguished by a number of factors such as the number of base pairs per turn of the helix, the pitch of the base pairs, and the handedness of the helical screw. There are three main families of double helical structures and these are the right handed A- and B-forms and the left handed Z-form.
Figure 1.3 A schematic representation of DNA [2].
Table 1.1 lists some of the differences between A-, B- and Z-DNA. Information on the various polymorphic variants of DNA may be found in references 5 - 9.

Table 1.1 Comparison of the structural properties of A-, B- and Z-DNA as derived from single crystal X-ray analysis [9].

<table>
<thead>
<tr>
<th>Property</th>
<th>A-DNA</th>
<th>B-DNA</th>
<th>Z-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall proportions</td>
<td>short and broad</td>
<td>longer and thinner</td>
<td>elongated and slim</td>
</tr>
<tr>
<td>Helix diameter</td>
<td>25.5 Å</td>
<td>23.7 Å</td>
<td>18.4 Å</td>
</tr>
<tr>
<td>Rise per base pair</td>
<td>2.3 Å</td>
<td>3.3 Å</td>
<td>3.8 Å</td>
</tr>
<tr>
<td>Sense of helix rotation</td>
<td>right handed</td>
<td>right handed</td>
<td>left handed</td>
</tr>
<tr>
<td>Number of base pairs per helix repeat</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Base pairs per helix turn</td>
<td>≈11</td>
<td>≈10</td>
<td>12</td>
</tr>
<tr>
<td>mean rotation per base pair</td>
<td>33.6 °</td>
<td>35.9 °</td>
<td>-30 °</td>
</tr>
<tr>
<td>Relative proportions of major groove</td>
<td>narrow and deep</td>
<td>wide and of intermediate depth</td>
<td>flattened out on helix surface</td>
</tr>
<tr>
<td>Relative proportions of minor groove</td>
<td>broad and shallow</td>
<td>narrow and of intermediate depth</td>
<td>very narrow and deep</td>
</tr>
</tbody>
</table>

The dominant DNA form in aqueous solution is the B form. The sugar phosphate strands in B-DNA project the hydrophilic phosphate groups out from the helix and the base pairs form a hydrophobic core along the DNA z axis. B-DNA has a helix diameter of 23.7 Å with
approximately 10 base pairs per turn of the helix, a distance of approximately 34 Å along the z axis. The base pairs are tilted approximately 1° from the plane perpendicular to the helix axis.

The base pair stacking sequence along the B-DNA z-axis produces major (broad) and minor (narrow) grooves which run the length of the macromolecule. The major groove is approximately 12 Å wide and 8.7 Å deep, and is on the side of DNA which contains the purine N-7. The minor groove, containing the pyrimidine O-2 and the purine N-3, is approximately 6 Å wide and 7.5 Å deep.

1.2 Ionizing Radiation

1.2.1 Radiation Sources

Radiation sources [10,11] commonly used in chemistry and biology may be divided into two groups; radioisotope sources and machine sources. These sources of radiation are described below.

Radioisotope Sources

Radioisotope sources produce α, β and γ radiation. Alpha particles are nuclei of helium atoms (He²⁺) and are spontaneously emitted with discrete energies by radioactive nuclei. Beta radiation consists of 'fast' electrons. In contrast to α particles, β radiation is emitted with energies ranging to a maximum characteristic of the element forming the radiation source.

Gamma rays are high energy electromagnetic radiation, emitted with energies characteristic of the source radioisotope. The two most widely used sources of γ radiation in research and industry are Co-60 and Cs-137. Co-60 is produced in metallic form by neutron activation of Co-59 whereas Cs-137 is separated from spent nuclear fuel. Cs-137 sources generally contain caesium as a chloride. Although the half life of Cs-137 (30.17 years) is longer than that of Co-60, most industrial and experimental radiation sources use Co-60 for two main reasons:
1. The energy of γ radiation from Co-60 (mean energy 1.25 MeV) is greater than that for Cs-137 (0.66 MeV). This higher energy leads to a more uniform dose distribution in the irradiated material.

2. Unlike Co-60, which is a metal, caesium chloride is water soluble and, should casing around a Cs-137 source become damaged, there is a risk of contamination, especially in irradiation facilities which use water as a radiation shield.

Cobalt-60 Radiation

Co-60 decays with a half life of 5.27 years, by β emission (predominantly via emission of β particles with an energy, E_{max} = 0.313 MeV) to yield Ni-60 in an excited state which instantaneously loses energy by releasing, in cascade, two gamma photons with energies of 1.173 and 1.333 MeV respectively (figure 1.4).

A Co-60 radiation source is generally manufactured using either pellets or thin disks of Co-60 which are placed into metal containers. These source elements are assembled into a radiation source of the desired size and configuration. The metal containment is sufficient to filter out the β radiation from the γ radiation, and thus a Co-60 source may be used to irradiate samples with γ radiation only.

1.2.2 Interaction Of Radiation With Matter

As radiation passes through matter, energy is transferred from the radiation to the surrounding medium. Radiation with mass and charge (α and β radiation) loses energy rapidly over relatively short distances by elastic and inelastic collisions. However electromagnetic ionising radiation (γ and X-rays) in general propagate through the material with infrequent interaction.
**Figure 1.4** Radioactive decay of Co-60

\[
\begin{align*}
^{60}_{27}\text{Co} & \rightarrow \beta, 99.8 \% \text{ (maximum energy 0.313 MeV)} \\
& \rightarrow ^{60}_{26}\text{Ni}^* \\
& \rightarrow \gamma, 1.173 \text{ MeV, 99.8 \%} \\
& \rightarrow ^{60}_{26}\text{Ni}^* \\
& \rightarrow \gamma, 1.333 \text{ MeV, 100 \%} \\
& \rightarrow ^{60}_{26}\text{Ni}
\end{align*}
\]
X-Ray and \( \gamma \) Radiation

X-rays and \( \gamma \) radiation interact primarily with electrons and, to a lesser extent, with nuclei of atoms of the target material. The nature of the interaction depends on the energy of the radiation and the composition of the material. The interaction processes are detailed below.

Coherent scattering

Incident photons can be deflected by interaction with the electron(s) of an atom. This coherent scattering forms the basis of X-ray crystallography where a well ordered material will diffract a collimated X-ray beam. However, in general this scattering is difficult to detect because the scattering angle is small and the incident radiation is rarely a "narrow beam".

Photoelectric effect

The photoelectric effect is the principal interaction process at low photon energies. In the photoelectric effect a photon ejects a single electron from an atom of the stopping material. In general an inner (core) electron is ejected. The resulting core-electron vacancy is filled by an outer-shell electron and the excess energy of the ion is dissipated by either X-ray emission or the ejection of one of the other electrons within the ion (an Auger electron) [10].
Compton scattering occurs when a photon interacts with an electron. The electron is accelerated and the photon is deflected with reduced energy. The energy of the "recoil electron" is equal to the energy of the incident photon less that of the deflected photon. In the γ irradiation of water, Compton scattering is the major interaction process for radiation with energy in the range of approximately 30 keV to 20 MeV [11].

Pair-production

The incident photon is completely absorbed in the vicinity of an atomic nucleus or an electron, followed by the emission of an electron and positron. The positron is slowed down by interaction with the target material and then annihilated by reaction with an electron to yield two γ photons with energies of 0.51 MeV. Pair production can only proceed for radiation with energy greater than 1.02 MeV [10].
The atomic absorption coefficients for water (figure 1.5) indicate the relative importance of pair production, photoelectric and Compton interaction processes at different photon energies. For Co-60 γ radiation (photon energies of 1.173 and 1.333 MeV) the principle interaction with water is by the Compton effect.

The Track Model

When α or β radiation interacts with matter, a trail of excited atoms, ions and molecules is produced along the path of the α or β particle. X-ray or γ radiation produces a similar effect because the energy deposited in the target medium is transferred initially to electrons which dissipate their energy in the same way as β radiation. Thus the general effect of radiation on matter is the formation of tracks of excited and ionised chemical species.

In radiation chemistry the different kinds of radiation yield similar products, but in different proportions. The differences in the amounts of products formed are due to α, β, X-ray and γ radiation dissipating energy at different rates and thus having different "track structures". The specific rate at which radiation deposits energy is defined as linear energy transfer (LET). Radiation with low LET, such as γ radiation, has a track structure which consists of spatially separated ions, electrons and excited molecules. These clusters are commonly referred to as spurs. With γ radiation, spurs are produced by secondary electrons. Radicals produced within a spur may either recombine or diffuse into the bulk solution and take part in further reactions. Models have been developed to simulate this process [12]. Samuel and Magee [13] have calculated that when γ photons interact with water the secondary electrons produce spurs with an initial diameter of approximately 2 nm and, on average, spurs in a γ photon track are separated by a distance of approximately 1 μm.
Figure 1.5 Atomic attenuation coefficients for water [11].

(A) Total attenuation coefficient, including coherent scattering; (B) photoelectric attenuation coefficient; (C) total Compton coefficient with coherent scattering; (D) total Compton coefficient without coherent scattering; (E) pair production coefficient [11].
1.2.3 Biological Systems

Irradiation of an aqueous suspension of cells results in cell inactivation which is attributed to radiation damage within the cell. Radiation damage to bulk water has little effect [14]. Microdosimetric studies in which eukaryotic cells are irradiated with radiation that penetrates to different depths show a drastic increase in lethal damage as the ionising radiation penetrates to the cell nucleus and hence to the DNA [15,16,17].

Irradiation of a biological system induces many chemical reactions. Radiation induced DNA damage is the most important process at the biological level because mutagenesis, carcinogenesis and reproductive cell death can all be related to radiation induced DNA damage [18,19]. Hence DNA is the main biological "target" for radiation damage.

Irradiation of DNA ultimately leads to several kinds of lesions such as base damage, single strand breaks and double strand breaks. As the name implies, in a single strand break one of the two ribophosphate chains in DNA is broken. The DNA strands in the vicinity of a single strand break may separate but in general the DNA molecule has to be thermally denatured, to "unzip" the double stranded DNA before fragmentation can be detected. A double strand break occurs when two single strand breaks are in close proximity and on opposite strands or when a single radiation induced event results in cleaving both DNA strands.

In order for DNA to function properly the base pair sequence must not be lost and so, in principle, any DNA lesion within a cell can be lethal. Cells possess repair mechanisms which efficiently counter DNA damage. In general DNA repair requires that one of the complimentary DNA strands is intact. Excision-resynthesis is the principle repair mechanism. A lesion in one DNA strand is recognised and a segment of the DNA chain which contains the lesion is removed. The missing segment is then re-synthesised using the intact complimentary chain as a template. Lesions in close proximity and on opposite strands are difficult to repair and double strand breaks correlate with cell lethality [20].
1.2.4 DNA Radiation Damage

Initial radiation induced DNA damage may be classed as being a consequence of either a direct effect or an indirect effect. In the direct effect absorption of the radiation energy by the DNA leads to the formation of radical cations and anions and excited states. The indirect effect gives rise to similar DNA damage, but arises from radicals formed by the radiolysis of the medium surrounding DNA. In vivo the relative contribution of the indirect effect and direct effect to overall DNA damage is difficult to assess but it has been estimated that approximately 50 - 70% is a result of the indirect effect and the remaining 30 - 50% is due to the direct effect [18].

The distinction between direct and indirect effects is not always clear. For example, absorption of radiation energy by DNA leads to electron loss and gain centres, as does the transfer of electrons or electron holes from DNA solvation water and DNA-bound proteins, such as histones. In general DNA solvation water and DNA-bound proteins are considered to be part of the "DNA target" and damage originating in the DNA target is classed as direct.

Frozen aqueous solutions of DNA provide a good in vitro model system with which to study initial radiation induced DNA damage. Aqueous solutions of DNA phase separate on freezing. The bulk solution forms ice-like water and radiation damage is trapped by the ice at low temperatures. On annealing, radicals in the bulk react amongst themselves without interacting with DNA. Whereas DNA and DNA solvation water form a separate phase, on freezing, which is glassy. Radiation damage in this glassy water phase can migrate efficiently to DNA [21,22,23]. This system is a good model for DNA radiation damage in vivo because, although approximately 70% of the mass of a cell is due to water, the DNA is generally located in the cell nucleus where it is folded around proteins and maintained as chromatin. Thus in vivo radiation damage to intracellular water is not necessarily transferred to DNA. However radiation damage to the water phase which solvates DNA is able to transfer radiation induced damage onto DNA.
Indirect DNA Damage

Radiolysis products of water are responsible for the majority of indirect radiation induced DNA damage. Of these radiolysis products the hydrogen atom, \( \text{H}^* \), the solvated electron, \( \text{e}^-(\text{aq}) \), and the hydroxyl radical, \( \text{OH}^* \), react with DNA. These products are formed by reactions 1 - 6 [18].

\[
\begin{align*}
\text{H}_2\text{O} + \gamma & \rightarrow \text{H}_2\text{O}^{++} + \text{e}^- & 1 \\
n\text{H}_2\text{O} + \text{e}^- & \rightarrow \text{e}^-(\text{aq}) & 2 \\
\text{H}_2\text{O}^{++} + \text{H}_2\text{O} & \rightarrow \text{H}_3\text{O}^+ + \text{OH}^- & 3 \\
\text{H}_3\text{O}^+ + \text{e}^-(\text{aq}) & \rightarrow \text{H}^* + \text{H}_2\text{O} & 4 \\
\text{H}_2\text{O} + \gamma & \rightarrow \text{H}_2\text{O}^* & 5 \\
\text{H}_2\text{O}^* & \rightarrow \text{H}^* + \text{OH}^- & 6 \\
\end{align*}
\]

Radicals and ions formed within the spur can react with one another, reactions 7 - 12, or diffuse into the bulk solution [18].

\[
\begin{align*}
\text{H}^* + \text{H}^* & \rightarrow \text{H}_2 & 7 \\
\text{e}^-(\text{aq}) + \text{e}^-(\text{aq}) + 2\text{H}_2\text{O}(\text{aq}) & \rightarrow 2\text{OH}^- + \text{H}_2 & 8 \\
\text{OH}^* + \text{OH}^* & \rightarrow \text{H}_2\text{O}_2 & 9 \\
\text{H}^* + \text{OH}^* & \rightarrow \text{H}_2\text{O} & 10 \\
\text{e}^-(\text{aq}) + \text{OH}^* & \rightarrow \text{OH}^- & 11 \\
\text{H}_3\text{O}^+ + \text{OH}^- & \rightarrow 2\text{H}_2\text{O} & 12 \\
\end{align*}
\]

The hydroxyl radical is the most reactive towards DNA [18]. The hydroxyl radical reacts with DNA either by abstracting hydrogen atoms or by addition to double bonds. The majority of \( \text{OH}^* \) add to the bases of DNA. Irradiation studies of solutions containing the nucleotides polyuridylic acid and polyadenylic acid have shown that base radicals can react with the sugar residue to produce a strand break [24].

The hydrated electron reacts almost exclusively with the DNA bases; reaction with the sugar-phosphate strands in DNA is negligible [18].
In solutions containing negatively charged dialkylphosphates the hydrated electron reacts slowly with the solute to break the C-O bond. In solutions containing purine or pyrimidine bases, hydrated electrons react by addition to form radical anions which are readily protonated by the solvent. Thus the reaction of the solvated electron with the DNA sugar-phosphate strands proceeds at a rate which is considerably less than that of addition to the bases.

The hydrogen atom is the least reactive of the water primary radiolysis products and its chemistry is similar to that of OH*. In oxygenated solutions the majority of hydrogen atoms and solvated electrons are converted into less reactive species; the hydroperoxy radical (HO₂*) and the superoxide radical ion (O₂*⁻), are formed by reactions 13 and 14. The hydroperoxy radical is the conjugate acid to the super oxide radical and the two are able to interconvert; reaction 15.

\[
e^- (aq) + O_2 \rightarrow O_2^{*-} \quad 13
\]
\[
H^* + O_2 \rightarrow HO_2^* \quad 14
\]
\[
H_3O^+ + O_2^{*-} \rightleftharpoons HO_2^* + H_2O \quad 15
\]

Both O₂*⁻ and HO₂* disproporionate to form hydrogen peroxide and oxygen; reactions 16 - 18.

\[
HO_2^* + HO_2^* \rightarrow H_2O_2 + O_2 \quad 16
\]
\[
HO_2^* + O_2^{*-} + H_3O^+ \rightarrow H_2O_2 + O_2 + H_2O \quad 17
\]
\[
O_2^{*-} + O_2^{*-} + 2H_3O^+ \rightarrow H_2O_2 + O_2 + 2H_2O \quad 18
\]

The contribution of e⁻ (aq) and H* to DNA damage via the indirect effect is minor in oxygen containing systems. However if transition metal ions are present in the system, OH* may be produced from the catalytic breakdown of H₂O₂ (reaction 19) and thus DNA damage may be enhanced [18].

\[
Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^* \quad 19
\]
Direct DNA Damage

In the direct effect, electron loss is indiscriminate [25]. However, DNA phosphate centred radicals such as (RO)₂PO₂⁺ and (RO)₂PO₂⁻ [26] and DNA sugar radicals have not been unambiguously detected and are thought to account for less than approximately 8% of the total radical yield. Electron spin resonance studies of frozen aqueous DNA solutions and of frozen films of hydrated DNA at low temperatures have shown that the electron loss centres are trapped at the purine bases (⁺G⁺ / ⁺A⁺), and the electron gain centres are trapped at the pyrimidine bases (⁻T⁻ / ⁻C⁻). Thus "primary" radical centres are not trapped even at 4 K. Electrons and electron holes migrate to the DNA bases and the absolute yields of ⁺G⁺, ⁺A⁺, ⁻T⁻ and ⁻C⁻ depends on the degree of DNA hydration and the dose rate [27,28]. There is no evidence for radicals trapped in pairs and the species detected by ESR must be separated by several base pairs. Proton transfer is the trapping mechanism that gives rise to base localised radicals [29].

ESR studies have shown that on annealing irradiated frozen aqueous DNA samples to approximately 190 K, the base radicals decay and the thymine radical protonated at the C-6 position is formed irreversibly. Wang et al [27] proposed a model for this direct process in which the initial effect of low LET radiation is the production of a spur. For hydrated DNA, at 77 K, approximately 75% of all radicals that initially form recombine within the spur, the remaining radicals being trapped. Electron holes that survive recombination migrate between the stacked DNA bases. Of the DNA bases, guanine has the lowest ionisation potential and thus the majority of electron holes are trapped at guanine.

Electrons which have escaped recombination transfer through DNA to the sites of highest electron affinity; cytosine and thymine. In hydrated DNA the electron affinities of cytosine and thymine are approximately equal [30] and the yields of cytosine and thymine radical anions (⁻T⁻ and ⁻C⁻) are approximately equal. At 77 K electron gain centres at cytosine are trapped by proton transfer from the complimentary guanine base. The cytosine radical anion, ⁻C⁻, is protonated at the N-3 position and this process, which traps the
radical in the protonated form ($\mathrm{^6C_H}$), is reversible. Annealing the sample to 130 K results in partial conversion of $\mathrm{^6T^-}$ to the more stable $\mathrm{^6C_H}$. At 190 K the irreversible protonation of $\mathrm{^6T^-}$ at the C-6 position yields $\mathrm{TH^6}$ [31]. Thus annealing frozen aqueous samples of DNA to 190 K converts the majority of electron gain centres into $\mathrm{TH^6}$. The proposed reaction scheme is summarised in figure 1.6.

1.3 Research Objectives

The aims of the research described in this thesis were to investigate the effects of ionising radiation on DNA and to understand the chemical basis of radiation damage to DNA. Initially DNA solvation in frozen aqueous solution was probed. The concept of a target for radiation induced DNA damage was developed and extended to examine how LiCl and NaCl in frozen aqueous solutions enhance total radiation induced DNA radical yields.

The interaction of sodium ions with DNA in (fluid) aqueous solution was investigated using NMR spectroscopy. Irradiation of solutions containing the sodium salt of DNA results in a narrowing of the Na-23 resonance. This line narrowing, in conjunction with polyelectrolyte theory as applied to DNA polyanions, was correlated with the release of sodium counter-ions from DNA following radiation induced single strand breaks.

1.3.1 Frozen Aqueous DNA Solutions

Irradiated frozen aqueous DNA samples are good model systems for DNA irradiated in vivo and can be studied using ESR spectroscopy. The initial aim of the research reported in this thesis was to investigate phase separation in frozen aqueous solutions, such as those used in ESR studies. Two phases are formed, ice-like water and glassy water. Ice-like water is formed from bulk water. Glassy water is the term used to describe water which is perturbed from the normal ice structure by interaction with DNA.
At 77 K both *T* and *C* form upon γ irradiation of DNA. At 77 K, *C* is reversibly protonated by its guanine base pair, forming *C_H*. Annealing to 130 K results in a partial conversion of *T* to the more stable *C_H* (indicated by the dashed arrows). At 190 K the electron gain centres are driven to form *TH* by an irreversible reaction [27].
In frozen aqueous solution the DNA radiation target comprises a glassy water and DNA phase. Chapter 2 discusses the amount of glassy water in frozen aqueous DNA samples. DNA solvation studies (for example reference 32) have determined that the primary DNA solvation layer comprises 21 - 26 water molecules per DNA base pair, whereas H-1 NMR studies [33] have shown that when bulk water is nucleated and forms ice, up to 200 water molecules remain mobile and are associated with the DNA. In general DNA solvation studies examine films of DNA in which the degree of DNA hydration is rigorously controlled. In contrast H-1 NMR studies examine solutions containing DNA.

The solvation of frozen DNA(aq) was examined by monitoring glassy water in solution using infrared spectroscopy. Using a solvent comprising HOD in D$_2$O rather than H$_2$O, the single O-H stretch band in the infrared is relatively sharp. However HOD molecules in glassy regions and in solvation sites contribute to a much broader feature. Thus band analysis of these narrow and broad bands leads to an assessment of the extent of phase separation. Information on the glassy water phase is necessary in order to gain an insight into the frozen system studied by ESR spectroscopy. Two objectives were: (i) to determine how much glassy water is present in the frozen system, and (ii) to describe the DNA radiation target in terms of solvating water.

**Salt Effects in Frozen DNA(aq) Solutions**

In the course of investigating the effect of buffers on the yield of single and double strand breaks in DNA [34], Symons *et al* examined the effects of 1:1 electrolytes on the yields of DNA radicals [35]. Some unusual salt effects were observed. Yields of single and double strand breaks measured the extent of overall DNA damage and ESR spectroscopy [22] measured the extent and form of the initial DNA damage.

ESR studies of frozen aqueous DNA solutions containing lithium or sodium chloride, irradiated at 77 K, showed a steady increase in the yield of DNA radicals as the concentration of salt was increased. The DNA radical concentration (at 130 K) for samples of salt (NaCl or
LiCl) solution having a concentration of 1 mol dm$^{-3}$ was approximately twice that for DNA solutions with no added salt. Added salt enhanced the yield of DNA electron gain centres more than electron loss centres.

The aim of the work reported in this thesis was to investigate phase separation in frozen aqueous DNA solutions containing salt (LiCl or NaCl). In particular, the study determined whether changes in the size of the glassy water-DNA-salt phase could be correlated with the increase in DNA radical yield as detected by ESR spectroscopy. The experiments explored the concept that the amount of ice-like water decreases and the amount of glassy water increases as the concentration of LiCl or NaCl increases. This concept is important because the products of radiation damage to ice-like water are trapped whereas the products of radiation damage to glassy water are not trapped efficiently and migrate to DNA thus leading to more DNA radiation damage. Thus if added LiCl or NaCl increases the amount of glassy water in the vicinity of DNA then the DNA radiation target is enlarged. The overall effect is an increase in the total target for DNA damage by gamma irradiation.

1.3.2 Detection Of DNA Strand Breaks

Bothe et al [36] showed that conductimetric measurements could be used to measure the number of strand breaks in polynucleotides and single stranded DNA. An increase in electrical conductance of DNA solutions following irradiation is attributed to the release of sodium counter-ions. In the final section of this thesis, Na-23 NMR line narrowing was used to confirm the results of Bothe et al [36] that approximately 15 sodium ions are released per strand break in single stranded DNA. The aim of the experiments was to determine whether Na-23 NMR line narrowing following the irradiation of native (double stranded) DNA correlated with DNA strand breaks.

When native (double stranded) DNA is irradiated, Na-23 NMR line narrowing (sodium ion release) is observed. This narrowing could be as a result of either single strand breaks or double strand breaks. The NMR technique enabled radiation induced Na-23 line narrowing to be interpreted in terms of DNA single strand breaks in native
DNA. Na-23 line narrowing was examined in conjunction with relationships describing dose yields of single and double strand breaks [37]. The resulting correlations demonstrate that Na-23 line narrowing is sensitive to single strand breaks but not double strand breaks, and that a single strand break releases approximately 8 sodium ions per DNA base pair. These experiments showed that NMR is an effective technique for measuring single strand breaks in both single stranded (denatured) DNA or double stranded (native) DNA. By way of contrast the conductimetric technique used by Bothe et al [36] could only be used to detect single strand breaks in polynucleotides and single stranded DNA.
1.4 References


Chapter 2

Infrared Studies Of Deoxyribonucleic Acid Hydration
Infrared Studies Of Deoxyribonucleic Acid Hydration

2.1 Introduction

This thesis is concerned with the effects of gamma radiation on DNA in frozen aqueous solutions. On freezing DNA solutions two phases are formed; ice-like water and glassy water. Ice-like water is formed from bulk solution. Glassy water is that which is prevented from forming an ice-like structure by interaction with DNA. Electron spin resonance studies [chapter 3, section 3.2.2] have shown that the "target" for radiation induced DNA damage comprises the phase containing DNA and approximately 30 glassy water molecules per DNA base pair.

The hydration of DNA has been the subject of many studies and a wide range of techniques have been used. Hydration numbers for DNA have been reported in the range 4 - 200 water molecules per DNA base pair. The aim of the research in this chapter was to determine a gross solvation number for frozen aqueous DNA, i.e. to assess the number of water molecules which form glassy water and do not form ice-like water.

The amount of glassy water per DNA base pair is compared to the radiation target which consists of DNA and 30 glassy water molecules per base pair. A model is proposed which describes the DNA radiation target in terms of hydration water.

This chapter reports infrared spectra of frozen aqueous DNA solutions. Using a solvent comprising HOD in D₂O rather than H₂O, the single O-H stretching band of ice-like HOD in the infrared is relatively sharp. However, HOD in glassy regions gives rise to a much broader infrared band. Thus band deconvolution, in which these broad and narrow bands are simulated, leads to an assessment of the extent of phase separation. The main objectives of this research were:
i. to determine the amount of glassy water present in frozen aqueous DNA solutions.

ii. to describe the DNA radiation target in terms of DNA solvation water.

The first objective was met by analysing infrared spectra of frozen aqueous DNA solutions of various concentrations. Infrared spectra were simulated using a broad band to represent glassy water and a narrow band to represent ice-like water. In order to achieve the second objective it was necessary to review the literature on DNA solvation. This review is presented in the following section.

2.2 Previous Work

2.2.1 Hydration of solid DNA films

In a series of papers, Falk and co-workers [1-4] reported gravimetric, infrared and ultraviolet spectroscopic studies of hydrated DNA films.

A gravimetric study [1] determined the number of water molecules bound to DNA as a function of relative humidity (r.h.) at 294 K. Application of a Brunauer Emmett and Teller (BET) adsorption isotherm was found to give an excellent fit to the experimental data below 80% r.h. Above 80% r.h., the data showed a sudden negative departure from the BET equation. The BET isotherm was used in the form:

\[ A = \frac{BCx}{(1-x+Cx)(1-x)} \]

where \( A \) is the total amount of water adsorbed at relative humidity \( x \), and \( B \) is the maximum amount of water accommodated on the primary adsorption sites of DNA. The constant, \( C \) is given by:

\[ C = \exp \left( \frac{E_1 - E_L}{RT} \right) \]
where \( R \) is the gas constant and \( T \) is temperature. The constants \( E_1 \) and \( E_L \) are the adsorption energies for the first and successive layers respectively. The adsorption energy for the first layer of adsorbed water was calculated as a variable to fit the experimental data. The energy of successive layers of adsorbed water was assumed to be equal to the energy of condensation of liquid water.

The BET parameters of best fit to water adsorption onto NaDNA were with \( B \) equal to 2.2 water molecules per nucleotide (4.4 water molecules per base pair) and \( C \) equal to 21. The constant \( C \) in the BET equation permitted a calculation of the energy of hydration of the first 4.4 water molecules per DNA base pair. Hence, 4.4 water molecules per DNA base pair were found to be bound to primary hydration sites with an energy of approximately 8.4 kJ mol\(^{-1}\) higher than that of subsequent water molecules. Comparison of adsorption isotherms of model DNA compounds (pyrimides, purines, nucleosides and nucleotides) showed that the primary hydration water of DNA is analogous to that of other ionic phosphate compounds. Primary hydration sites on DNA were thus identified as the phosphate groups (\( R_2PO_2^- \text{Na}^+ \)).

The deviation from the BET isotherm above 80% r.h. was interpreted as being due to incoming water molecules forcing DNA molecules apart. This expansion would require an input of energy and would account for the negative deviation from the BET model.

In their second paper of the series, Falk and co-workers [2] used infrared spectroscopy to determine the sites of hydration on DNA. Infrared spectra of DNA films were studied as a function of r.h. From frequency and intensity changes of infrared bands, it was concluded that the phosphate group (\( R_2PO_2^- \text{Na}^+ \)) becomes hydrated in the 0 - 65% r.h. range (approximately 0 - 15 water molecules per DNA base pair). In the range 60 - 75% r.h. (approximately 12 - 18 water molecules per DNA base pair) the DNA undergoes a conformational change, and in the range 75 - 80% r.h. (approximately 18 - 22 water molecules per DNA base pair) the DNA bases become hydrated at the carbonyl and ring nitrogen groups.
In their third paper, Falk and co-workers [3] examined the effect of hydration on the structure of DNA. Polarised infrared and ultraviolet spectra of oriented DNA films were investigated between 0 and 92% r.h. Absorbances were measured with the electric field vector of polarised light in a plane parallel (An) and perpendicular (Aj) to the z-axis of the DNA fibres. Plots of the dichroic ratio (An/Aj) of an infrared band at 1660 cm⁻¹ (attributed to carbonyl and ring nitrogen groups of the DNA bases) against r.h. showed a transition between conformational forms in the range 55 - 75% r.h. (corresponding to approximately 10 - 18 water molecules per DNA base pair). Similar results were obtained with the dichroic ratio of the ultra-violet band at 260 nm. These results were interpreted as being due to disordered DNA adopting the B configuration upon hydration. At relative humidities greater than 75% (i.e. approximately 18 water molecules per DNA base pair) no further transition was observed, indicating that B-DNA is the dominant conformation of hydrated DNA.

In a later paper [4] Falk et al studied the state of DNA hydration water using infrared spectroscopy. One of the aims of this study was to detect "crystallisation" of part of the DNA hydration shell and to establish the nature of the water phase or phases present at low temperature. Infrared spectroscopy was used to examine the solvating water over the temperature range 143 - 298 K. The O-D stretching mode of water (isotopically dilute HOD in H₂O) was monitored on cooling.

The degree of DNA solvation was controlled by maintaining partially deuterated films of DNA at constant relative humidity prior to cooling. A single broad O-D stretching band was observed with samples having up to 10 water molecules adsorbed per DNA nucleotide (i.e. 20 water molecules per DNA base pair). As well as the broad feature, a second infrared band was detected in samples that contained greater than 10 water molecules per DNA nucleotide. This second component in the O-D stretching region overlapped the broad band and was narrow. The full width at half height of the narrow infrared band was similar to that of O-D in ice. DNA films with 20 - 26 water molecules per base pair only gave rise to this ice-
like band after being kept at dry ice temperatures for several days. It was concluded that 20 water molecules per DNA base pair formed an inner hydration layer that is "incapable of crystallisation". A further 6 water molecules per base pair "crystallised" with difficulty to form an intermediate solvation layer. Above 26 water molecules per base pair the hydration water adopted a more ice-like structure.

Brandes et al [5] employed high resolution deuterium NMR spectroscopy to investigate the interaction of water with oriented DNA fibres. In the liquid state, nuclear spin dipole - dipole interactions average to zero. However, water adsorbed onto solid DNA is preferentially oriented and the dipolar interactions of H-2 nuclei of water molecules do not average to zero. This incomplete averaging of the dipolar interactions produces a doublet spectrum. The difference in frequency which separates the two resonance bands of the doublet (the doublet splitting) is sensitive to the average order of the water molecules with respect to the DNA molecule. Variations in the observed splitting of the deuterium doublet with hydration were studied for both lithium and sodium DNA salts.

**LiDNA**

At approximately 5 D$_2$O molecules adsorbed per DNA base pair, a broad singlet resonance was observed. At higher levels of hydration the H-2 resonance was a doublet. The maximum doublet splitting (approximately 3 - 4 kHz) was at approximately 10 D$_2$O molecules per DNA base pair. In the range 10 - 14 D$_2$O molecules per LiDNA base pair, additional hydration water rapidly reduced the doublet splitting. In the range 14 - 24 D$_2$O molecules per LiDNA base pair the doublet splitting was approximately constant. Between approximately 24 and 43 D$_2$O molecules per LiDNA base pair the reduction in the doublet splitting with adsorbed water was rapid and approximately linear. Above approximately 43 D$_2$O molecules per base pair the rate of decrease in the doublet splitting, with additional water, was reduced.

These results, for LiDNA, were interpreted as follows: DNA phosphodiester groups become hydrated in the range 0 - 10 D$_2$O molecules per DNA base pair. At approximately 10 adsorbed water
molecules per DNA base pair the DNA undergoes a conformational
transition to produce B-DNA. Water molecules are adsorbed into the
B-DNA primary solvation sites in the range 12 - 24 $D_2O$ molecules
per DNA base pair, the primary hydration layer being filled at
approximately 24 $D_2O$ molecules per base pair. Additional hydration
water adds to the secondary hydration layer, this secondary
hydration layer was filled at approximately 43 $D_2O$ molecules per
DNA base pair.

**NaDNA**

In the low hydration range (0 - 18 $D_2O$ molecules per DNA base pair)
the H-2 NMR spectra obtained from three separate preparations of
NaDNA were not the same. Preparation I did not produce a H-2
NMR doublet resonance until approximately 20 $D_2O$ molecules were
adsorbed per NaDNA base pair. In contrast, preparations II and III
did give rise to a H-2 doublet when hydrated to approximately 10
and 14 $D_2O$ molecules per base pair, respectively. At 20 water
molecules per DNA base pair, DNA is in the B- form [3]. All three
NaDNA preparations gave rise to a H-2 NMR doublet with similar
splittings when hydrated to greater than approximately 20 $D_2O$
molecules per DNA base pair.

The differences between H-2 NMR spectra of NaDNA samples at low
levels of hydration were interpreted in terms of each preparation
containing DNA in both A- and B- forms. The proportion of A-DNA
and B-DNA was considered to be different in each NaDNA
preparation.

**2.2.2 Hydration Of Frozen Aqueous DNA**

Proton spin echo NMR spectroscopy was utilised by Lubas and
Wilczok [6] to detect "non-rotationally bound water" associated with
DNA in aqueous solutions. Non-rotationally bound water was defined
as part of the DNA hydration water which contained protons which
strongly interact with DNA such that water dipoles do not have
freedom of movement in the high-frequency field. All other water
molecules were assumed to have relaxation times $T_1$ and $T_2$ similar
to those of pure water. Non-rotationally bound water and other water molecules were considered to be in fast exchange.

Lubas and Wilczok reported approximately 0.090 to 0.104 g of non-rotationally bound water molecules per gram DNA (i.e. 3.3 - 3.8 water molecules per DNA base pair). This is in good agreement with the 4.4 water molecules per base pair calculated as the most strongly "DNA-bound" water molecules by Falk et al [1].

Kuntz et al [7] employed low temperature proton NMR to determine the hydration number of several biomolecules. The complication of rapid proton exchange between bulk and solvation water was avoided by studying frozen aqueous H2O solutions. In the correct temperature regime, hydration water remains mobile on the NMR time scale, whereas bulk water is frozen and held immobile as ice. At temperatures as low as 238 K, proton resonance is observed in solutions of proteins and other biomolecules.

Ice does not exhibit proton resonance in conventional NMR spectroscopy. On cooling, the proton signal from bulk water broadens and, once frozen, the broad signal cannot be distinguished from the baseline. However, with proton NMR of frozen aqueous solutions which contain macromolecules, proton resonance is observed due to a fraction of the water which remains mobile. The integrated "unfrozen" water proton resonance is directly proportional to the macromolecule concentration. The unfrozen water is part of the hydration sheath surrounding the macromolecule and is prevented from forming ice-like water.

Mathur-De Vre and co-workers [8] used low temperature NMR spectroscopy to investigate DNA and polynucleotide hydration. DNA hydration was calculated as grams of hydration water per 100 gram weight of DNA (H%). The ratio of unfrozen water to total water was calculated from the integrated proton resonance of water protons at +5 °C and at -5 °C (labelled Δ+5 and Δ-5 respectively). The integral Δ+5 was directly proportional to the total number of water protons, and similarly Δ-5 corresponded to the amount of unfrozen, solvation, water molecules associated with the macromolecule. A plot of \( \frac{\Delta-5}{\Delta+5} \) against macromolecule concentration resulted in a
straight line with the origin as its intercept. DNA was found to be hydrated by approximately 200 g of unfrozen water per 100 g DNA (approximately 74 water molecules per DNA base pair).

In a more recent study, Mortimer [9] employed proton NMR to study the "non-frozen" (mobile) water associated with DNA in frozen aqueous solution. Proton NMR measurements were made at a frequency of 59.4 MHz using a Bruker CXP 100b NMR spectrometer. Free induction decays (FIDs) were measured with signal averaging following a $P_x(90^\circ)$ pulse. Temperature control ($\pm 1.0$ K) was achieved using a conventional gas-flow system.

At 200 K the proton FIDs for an aqueous DNA solution and a sample of pure water were similar, i.e. the signal was that of frozen water. On increasing the temperature to 210 - 215 K a second component in the proton NMR became apparent. At 225 K this feature could be resolved; the decay was exponential with a $T_2$ value of 55 ± 10 $\mu$s and was attributed to non-frozen (mobile) water. With a further increase in temperature there was a marked lengthening of the proton NMR FID due to this non-frozen water. At 270 K, just prior to melting, there was a distinct increase in signal amplitude.

At all temperatures above 225 K, the proton NMR FID signal for frozen DNA solutions consisted of ice-like and non-ice-like components. The time decay of the ice component was rapid and this enabled the signal amplitude of the non-ice-like component to be determined by extrapolation to zero time. Table 2.1 gives the signal amplitude of the non-ice like component as a percentage of the FID amplitude measured at 293 K. The FID amplitude extrapolated to zero time is proportional to the amount of un-frozen water; corrections were made for the Curie Law dependence on temperature [10].

Mortimer's results show that approximately 11% - 13% of the water in solution is non-frozen (i.e. mobile) at 225 to 245 K. The DNA solution consisted of 25 mg DNA added to 1 cm$^3$ H$_2$O. Thus, approximately 162 - 190 non-frozen water molecules were detected per DNA base pair.
Table 2.1  The effect of temperature on the proton NMR FID signal of non-frozen water in aqueous DNA solution [9].

<table>
<thead>
<tr>
<th>Temperature /K</th>
<th>Corrected relative amplitude / %†</th>
</tr>
</thead>
<tbody>
<tr>
<td>225.0</td>
<td>11</td>
</tr>
<tr>
<td>245.0</td>
<td>13</td>
</tr>
<tr>
<td>260.0</td>
<td>19</td>
</tr>
<tr>
<td>269.5</td>
<td>25</td>
</tr>
<tr>
<td>293.0</td>
<td>100</td>
</tr>
</tbody>
</table>

† The amplitude of the FID signal extrapolated to zero time, as a percentage of the amplitude at 293 K, estimated error ±5%.

Archer [11], in an NMR experiment similar to that of Mathur-De Vre et al [8], probed DNA hydration in frozen aqueous solution. The experiment measured the integrated water proton resonance of DNA solutions as they were cooled to 233 K.

The integrated water proton resonances of DNA solutions were compared to an internal reference (a solution of tetramethylsilane, TMS, in deuterated trichloromethane) which was sealed in a 5 mm o.d. NMR tube and held within a 10 mm o.d. NMR tube that contained the DNA solution. The proton NMR spectrum was recorded at 278 K. The resulting water and TMS peaks were integrated and their relative values compared. The sample was cooled to the required temperature and the proton NMR spectrum recorded. The resulting water and TMS peaks were integrated and their relative values again compared. Thus the concentration of unfrozen water at a given temperature (provided by the integral of the proton water resonance) could be calculated by comparison with the integral of the TMS peak. The TMS peak effectively remained constant over the temperature range studied.

The amount of unfrozen water was determined at 261 K. Archer observed that the mobile water phase consisted of approximately 25 water molecules per DNA base pair. This is surprisingly small by comparison with the results of Mathur-De Vre et al [8] and Mortimer [9].
2.2.3 Theoretical And Crystallographic Methods

Alden and Kim calculated the solvent accessible surface areas to nucleic acids [12]. A variety of polynucleotides were examined including DNA. Their calculations assumed a spherical water 'probe' molecule with a radius $r_w$ (equal to 1.40 Å) which was free to touch, but not penetrate, the Van der Waals surface of the examined molecule. The closed surface defined by all possible loci for the centre of the probe was defined as the accessible surface of the molecule under investigation. A computer program was used to calculate the envelope defining the loci of points which defines the solvent accessibility.

Alden and Kim calculated the Van der Waals solvation surface of B-DNA as 450 Å³ per DNA base pair. Dividing this volume by the mean volume of one water molecule (30 Å³) gives 15 water molecules per base pair immediately adjacent to B-DNA. The DNA groove volumes were estimated by enclosing a model of B-DNA in a cylinder. The amount of water enclosed by the DNA grooves and Van der Waals surface was estimated as 38.6 water molecules per DNA base pair. This number of water molecules is in good agreement with that of approximately 43 water molecules per DNA base pair required to complete the primary and secondary hydration layers in the H-2 NMR study of Brandes et al [5].

The oligonucleotide d(CGCGAATTCCGCG)

The dodecamer duplex d(CGCGAATTCCGCG) was the first oligonucleotide found to crystallise in the B form and has been subjected to both X-ray crystallographic and theoretical studies probing the position of hydration water.

Single crystal X-ray crystallography of this dodecamer, by Dickerson et al [13] showed the position of crystallographically organised hydration water on the atomic scale. Between one and three water molecules were found near most phosphate oxygen atoms; very few were detected close to the phosphate di-ester oxygen atoms. A chain of nine water molecules was seen to extend along the minor
The primary hydration shell became less well ordered toward the ends of the helix.

The issues involved in locating solvent peaks on nucleic acid hydrates were critically considered in a re-refinement of the room temperature structure of the dodecamer by Westhof [14]. The chain of water molecules along the minor groove was found to be conserved on refinement. In a review of the hydration of oligonucleotides in crystals [15], Westhof analysed solvent molecules in terms of interaction sites with bases, phosphates and sugars. It was concluded that, through the periodicity of the nucleic acid structure, water molecules may bridge hydrophilic atoms and that such bridges may lead to hydration networks involving several water molecules. These 'spines of hydration' were viewed as dynamic hydration patterns which occur frequently in the aqueous hydration of nucleic acids.

Subramanian and Beveridge [16] produced a theoretical study of the hydration of the d(CGCGAATTCGCG) duplex in a computer simulation. The simulation modelled hydration of this duplex dodecamer in aqueous solution, interacting with 1777 water molecules. Counter-ion screening of nucleic acid charge was modelled by uniformly reducing the nucleic acid charge by 75%. The simulation was allowed to run for 1.5 million configurations of "equilibration" followed by a further 2.5 million configurations of "production" where the results were analysed.

Water molecules found within contact radii of atoms of the duplex dodecamer were assigned as the first co-ordination shell. This approach permitted the degree of hydration to be assessed in terms of the minor groove, major groove, phosphate and sugar components of the macromolecule.

Computer modelling showed that the inner (primary) solvation shell consisted of approximately 21 water molecules per base pair; 11 water molecules per base pair were associated with each phosphate group and 6 water molecules per base pair were associated with each sugar moiety. The minor groove contained 1 water molecule per
base pair, and 3 water molecules per base pair were contained within the major groove.

Secondary solvation was found to consist of a further 87 water molecules per base pair. Approximately 47 of these water molecules were assigned to the anionic oxygen atoms of the phosphate groups, 26 were assigned to the sugar moiety and the minor and major grooves accommodated 3 and 11 water molecules per base pair respectively. The calculated hydration density associated with sugar groups on the DNA backbone was considerably more diffuse than that observed for either the phosphates or the major and minor grooves. Cones of hydration were observed to be near to the phosphate anionic oxygen atoms.

2.2.4 Summary Of Previous Work

The amount of DNA hydration water as reported by different groups is summarised in table 2.2. There are discrepancies in the number of water molecules assigned as DNA solvation water. The range of solvation numbers reported reflects the wide range of techniques used to detect solvating water associated with DNA. Each technique inherently sets limitations on the water which may be detected and this gives rise to different values for the number of water molecules solvating DNA.

The DNA hydration numbers given in table 2.2 are consistent with phosphate groups on the DNA backbone being solvated by approximately 4 water molecules per DNA base pair. The DNA primary solvation layer consists of 21 - 26 water molecules per base pair, and approximately 40 water molecules per DNA base pair correspond to an intact DNA primary solvation layer plus completely filled major and minor grooves.
Table 2.2 Summary of DNA hydration as determined by various authors.

<table>
<thead>
<tr>
<th>Reference</th>
<th>DNA hydration (water molecules per DNA base pair)</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Falk et al [1]</td>
<td>4.4 (primary)</td>
<td>Gravimetric study of solid DNA films</td>
</tr>
<tr>
<td>Falk et al [4]</td>
<td>20 (inner layer) 26 (inner + intermediate solvation)</td>
<td>Infrared study of solid DNA films</td>
</tr>
<tr>
<td>Brandes et al [5]</td>
<td>24 (primary layer) = 43 (primary + secondary layers)</td>
<td>Deuterium NMR of solid DNA films</td>
</tr>
<tr>
<td>Lubas et al [6]</td>
<td>3.3 - 3.8</td>
<td>H-1 NMR of liquid aqueous DNA solutions</td>
</tr>
<tr>
<td>Mathur de Vre et al [8]</td>
<td>74</td>
<td>H-1 NMR of frozen aqueous DNA solutions</td>
</tr>
<tr>
<td>Mortimer [9]</td>
<td>162 - 190</td>
<td>H-1 NMR of frozen aqueous DNA solutions</td>
</tr>
<tr>
<td>Alden et al [12]</td>
<td>15 adjacent = 39 (adjacent + filled DNA groove volumes)</td>
<td>Calculation of solvent accessible surface</td>
</tr>
<tr>
<td>Subramanian et al [16]</td>
<td>21 (primary layer) 108 (primary + secondary layers)</td>
<td>Monte Carlo Simulation</td>
</tr>
</tbody>
</table>
The large amount of DNA solvation water calculated by Mortimer [9] and Mathur de Vre et al [8] are "gross" solvation numbers for DNA in aqueous solution. These gross solvation numbers are estimates of the maximum solvation i.e. solvating water is defined as the mobile fraction of water in frozen aqueous DNA solutions. For this reason, it is surprising that Archer [11] detected only 25 non-frozen water molecules per DNA base pair. All three NMR studies of frozen aqueous DNA solutions should result in large amounts of hydration water per DNA base pair.

2.3 Experimental

2.3.1 Materials

Highly polymerised, sodium salt of calf thymus DNA was obtained from BDH Chemical Limited. Deuterium oxide (D₂O 99.9% D) was obtained from Goss Limited.

2.3.2 Procedure

Sample preparation

As purchased, DNA contains excess sodium chloride. Prior to sample preparation sodium chloride was removed by dialysis. The dialysis procedure involved placing a DNA solution into cellulose dialysis tubing. The solution consisted of approximately 500 mg DNA added to 25 cm³ of water. This solution was dialysed against water at 277 K for approximately 24 hours. DNA was recovered by freeze drying.

DNA samples were prepared by adding a known weight of NaDNA to a known volume of deuterated water (D₂O). Samples were left to fully hydrate at 277 K for at least 24 hours before use.

Infrared spectroscopy

Spectra were recorded using a Perkin Elmer 681 double beam spectrometer. The sample was placed in a cell sample holder which was sealed. The cell consisted of two zinc selenide plates separated
by a $2.5 \times 10^{-3}$ cm spacer. The sample cell was locked into a vacuum jacket and cooled to the required temperature. Nitrogen gas was bled into the vacuum jacket to help prevent water condensing on the infrared cell windows. Samples were cooled to approximately 208 K using liquid nitrogen as the coolant. This coolant was then replaced by a dry ice / methanol bath and the temperature of the sample was slowly raised to 218 K under thermostatic control. Infrared spectra were recorded at 218 K.

**Absorption band simulations**

Infrared spectra were normalised by dividing the absorbance recorded at each wavenumber by the maximum observed absorbance. Thus, the maximum absorbance is 1.00 for a series of normalised spectra. Normalised spectra were simulated using a spreadsheet computer package (Microsoft Excel). Simulations were produced by generating curves centred at various wavenumbers, each one representing an infrared absorption. These curves were summed to simulate the actual infrared absorption envelope in the O-H stretching region of isotopically dilute HOD. Simulated spectra were plotted over infrared spectra in the O-H stretching region of HOD in frozen aqueous (D$_2$O) solutions of DNA.

**2.4 Results**

A plot of the full width at half peak height (FWHH) of the O-H absorption band of HOD (centred at 3300 cm$^{-1}$) is given in figure 2.1. Increasing amounts of DNA broaden the O-H absorption band in frozen aqueous (D$_2$O) solution. Linear regression of the FWHH data for frozen aqueous DNA samples in figure 2.1 yields a straight line with an intercept of approximately 3 cm$^{-1}$ less than the FWHH of frozen water (a frozen solution of isotopically dilute HOD in D$_2$O). Thus, in the absence of DNA, a frozen water solution produces an O-H absorption band with a FWHH greater than that recorded for solutions containing up to 10 mg DNA.
Figure 2.1: Full width at half height (FWHH) of the O-H stretching mode of HOD in frozen aqueous (D2O) solutions containing increasing amounts of DNA. Infrared spectra were recorded at 218 K.
Infrared spectra were simulated by combining a Gaussian band with the infrared O-H absorption band of a frozen solution containing 2.5 mg DNA. The Gaussian band represents the contribution of the glassy water to the spectra. The absorption due to ice-like water was simulated using the infrared absorption band for a frozen solution containing 2.5 mg DNA.

To simulate ice-like water it was originally intended to use an infrared spectrum of O-H in frozen (D$_2$O) water. However the FWHH of the O-H band in frozen water is greater than the FWHH of solutions prepared by adding up to 10 mg DNA to 1 cm$^3$ D$_2$O. Thus the infrared absorption band of O-H in frozen D$_2$O solution, does not give a good fit to real spectra. The relatively narrow O-H stretch infrared band obtained from the solution with lowest DNA concentration (2.5 mg added to 1 cm$^3$ of D$_2$O) was used to simulate ice-like (bulk) water.

The parameters for the Gaussian band were determined by subtracting infrared spectra (between 3100 cm$^{-1}$ and 3500 cm$^{-1}$) of solutions containing different amounts of DNA. Infrared spectra of solutions having low DNA concentrations were subtracted from infrared spectra of samples having higher DNA concentrations. This procedure yielded a Gaussian band having a FWHH in the range 90 cm$^{-1}$ - 100 cm$^{-1}$ with a maximum absorption centred at approximately 3300 cm$^{-1}$.

In the simulations, a best fit to 'real' spectra was achieved using a Gaussian band (representing glassy water) with a FWHH of 95 cm$^{-1}$ centred at 3396 cm$^{-1}$ in conjunction with the infrared spectrum of a frozen solution containing 2.5 mg DNA (representing ice-like water). These two bands were summed together and the resulting band shape fitted to the 'real' spectrum by eye. The Gaussian simulation band and the O-H absorption band in D$_2$O were scaled independently, until their sum matched that of the real spectrum under investigation. Simulated and observed spectra are given in appendix A figures A1 to A5.

For each infrared spectrum, the relative amount of glassy HOD in solution could be determined as a fraction of total HOD present by
calculating the area beneath the simulated infrared absorption due to glassy HOD. Dividing the area beneath the Gaussian band (A\text{Gaussian}) by the area beneath the total O-H absorption envelope of the real spectrum (A\text{real}) yields the fraction of glassy water present.

\[
\text{Glassy water~\%} = \left( \frac{A\text{Gaussian}}{A\text{real}} \right) \times 100
\]

The area A\text{2.5mg} was also calculated; this is the area beneath the infrared absorption band which represents ice-like water (i.e. an infrared spectrum of a frozen solution containing 2.5mg DNA). In appendix A figures A1 to A5, the area A\text{2.5mg} is the integral of band C, A\text{Gaussian} is the integral of band D and A\text{real} is the area beneath band B.

The areas, A\text{Gaussian}, A\text{real} and A\text{2.5mg} for each simulated spectrum are given in table 2.3. The areas beneath these three bands were calculated by integrating the appropriate infrared band between 3200 cm\(^{-1}\) and 3400 cm\(^{-1}\).

When calculating the percentage glass content by A\text{Gaussian}/A\text{real}, an assumption was made that the extinction coefficients of O-H oscillators in ice-like water and in glassy water are the same. This assumption is supported by the simulated spectra which showed that the gain of intensity in the glassy water simulation band was equal to the loss in intensity of the simulated infrared O-H absorption band associated with ice-like water. The amount of glassy water was calculated assuming that the ratio of glassy HOD to ice-like HOD was equal to the ratio of glassy water (glassy HOD + glassy D\text{2O}) to ice-like water (ice-like HOD + ice-like D\text{2O}).
Table 2.3 Integration of the observed, "real", O-H stretching infrared absorption band and also of the best fit simulation bands. The bands were integrated between 3200 cm\(^{-1}\) and 3400 cm\(^{-1}\).

<table>
<thead>
<tr>
<th>Mass of DNA added to 1 cm(^3) D(_2)O / mg</th>
<th>A_Gaussian / cm(^{-1})</th>
<th>A_2.5mg / cm(^{-1})</th>
<th>A_real / cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Integral of the Gaussian band</td>
<td>Integral of the 2.5 mg DNA + 1 cm(^3) D(_2)O solution scaled to fit spectra.</td>
<td>Integral of the 'real' spectrum</td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td>53.53</td>
<td>53.53</td>
</tr>
<tr>
<td>5</td>
<td>1.99</td>
<td>52.18</td>
<td>57.72</td>
</tr>
<tr>
<td>10</td>
<td>2.98</td>
<td>51.90</td>
<td>56.32</td>
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<tr>
<td>15</td>
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<tr>
<td>20</td>
<td>5.98</td>
<td>47.77</td>
<td>59.23</td>
</tr>
<tr>
<td>50</td>
<td>19.11</td>
<td>43.63</td>
<td>67.58</td>
</tr>
</tbody>
</table>

**Glassy water per DNA base pair**

The percentage glassy water in frozen aqueous DNA solution increases linearly with DNA content (Figure 2.2). In order to demonstrate the validity of the simulation, data calculated by dividing A_Gaussian by A_simulation (A_simulation = A_Gaussian + A_2.5mg) have also been plotted in figure 2.2. The percentage glassy water calculated by A_Gaussian/A_real are generally within a few percent of those calculated by A_Gaussian/A_simulation. Where the two values differ, that given by A_Gaussian/A_real is always greater than that given by A_Gaussian/A_simulation.

Linear regression of A_Gaussian/A_real data in figure 2.2 was performed with the following criterion: the regression line passes through A_Gaussian/A_real = 0\% at the position on the x axis corresponding to 2.5 mg DNA added to 1 cm\(^3\) D\(_2\)O. This constraint
Figure 2.2 Amount of glassy water as a percent of total (glassy and ice-like) water in frozen aqueous solutions of DNA.

![Graph showing the amount of glassy water as a percent of total water in frozen aqueous solutions of DNA. The graph is a scatter plot with data points indicating the relationship between the mass of DNA added to 1 cm$^3$ D2O and the amount of glassy water. The line of best fit is shown with a gradient of 0.59% mg$^{-1}$ and an intercept of -1.50%.]
on the regression is necessary because the O-H absorption band observed with a sample prepared by adding 2.5 mg DNA to 1 cm$^3$ D$_2$O was used, in the simulations, to correspond to the infrared absorption band of O-H in ice-like water. Thus, by definition, this point is fixed and the regression line must pass through it. The gradient of the line determines the number of glassy water molecules per DNA base pair and not the intercept, thus the procedure described above is valid.

The gradient of the regression line gives 0.59 (±0.05)% glassy water molecules per mg DNA. Samples were prepared by adding a known mass of DNA to 1 cm$^3$ D$_2$O. Thus 0.59 (±0.05)% glassy water molecules per mg DNA (RMM 662.15 mol DNA base pairs g$^{-1}$) corresponds to 212 (± 20) D$_2$O molecules per DNA base pair.

2.5 Discussion

In frozen aqueous solutions containing DNA there are approximately 212 glassy water molecules per DNA base pair. This solvation number is very large by comparison with those reported in table 2.2. Such a large solvation number reflects the definition of solvation adopted in this study. Solvating water quantified here refers to glassy water i.e. that water which is perturbed from forming ice-like water by interaction with DNA and forms a glassy water phase.

A model for frozen DNA(aq) solvation

The extent of the glassy water phase indicates that a solvated DNA molecule has a large influence on the water structure in frozen aqueous solution. Water which is not associated with DNA (bulk water) forms ice-like water on freezing, whereas water closely associated with DNA forms a glassy phase which nucleates at a lower temperature than bulk water [8, 9].

The glassy water phase consists of water which bridges between DNA and the ice-like phase. Water becomes progressively more ice-like the further it lies from the DNA molecule. Studies of solid DNA films [4, 5] indicate that the primary hydration of DNA consists of between
21 to 26 water molecules per DNA base pair. Approximately 40 water molecules per base pair form the primary hydration layer and fill the DNA groove volumes [5, 12]. NMR studies [8, 9] show that between 75 and approximately 162 to 190 water molecules remain mobile at low temperatures and are prevented from forming ice-like water by interaction with DNA. Hence one could describe water in a frozen aqueous DNA solution according to a three zone model; zone A is DNA solvation water; zone C is water in bulk solution and zone B consists of water which bridges these two phases. This three zone model is shown schematically in figure 2.3.

Zone A represents all those water molecules which are nearest neighbours to the DNA molecule and which make up the primary or inner solvation sheath. Zone C represents the unperturbed, ice-like water molecules in bulk solution. Zone B results from the incompatibility of the hydrogen bonding regimes between water of the bulk solution (Zone C) and the primary solvating sheath (Zone A). Zone B is a "mismatch" region which does not have clearly defined boundaries, but consists of water molecules which show a gradual progression from being "zone A like" to being "zone C like" in structure.

Relative humidity (r.h.) studies of solid DNA films, such as those undertaken by Falk et al [1-4] and Brandes et al [5], detect zone A water. Thus, with B-DNA, zone A consists of approximately 21 - 26 water molecules per base pair.

In this investigation into DNA hydration, the amount of zone A and zone B water has been assessed using infrared spectroscopy to monitor glassy water in frozen aqueous DNA solutions. The amount of water molecules belonging to zone A and zone B has been calculated as approximately 212 water molecules per DNA base pair. This result is in good agreement with a recent NMR experiment [9] which calculated that approximately 162 to 190 water molecules per DNA base pair remain mobile in frozen aqueous DNA solutions between 225 and 245 K.
Figure 2.3 Schematic representation of the three zone model of DNA solvation water.

Zone A is the inner or primary solvation water, zone C is bulk (ice-like) water and zone B is water which bridges between water of zone A and C.

In the following chapter the radiation target for DNA damage in frozen aqueous DNA solution is discussed. This radiation target comprises the phase containing DNA and approximately 30 glassy water molecules per DNA base pair (chapter 3, section 3.2.2). In terms of DNA solvation this target contains zone A water with a small contribution from zone B water. The radiation target is larger than DNA plus a primary solvation layer of 21 - 26 water molecules per base pair, but is smaller than approximately 40 water molecules per base pair which represent a primary DNA solvation layer plus DNA "groove water" i.e. water which fills the major and minor groove volumes.
Appendix A

Infrared Spectra of DNA(aq)

Simulated and "real" infrared spectra of frozen aqueous solutions containing DNA (figures A1 - A5).
Figure A1  Simulation of the infrared O-H absorption band of HOD in frozen aqueous (D2O) solution at 218 K. The sample was prepared by adding 5 mg DNA to 1 cm3 of D2O.
Figure A2. Simulation of the infrared O-H absorption band of HOD in frozen aqueous (D2O) solution at 218 K. The sample was prepared by adding 10 mg DNA to 1 cm3 of D2O.
Figure A3  Simulation of the infrared O-H absorption band of HOD in frozen aqueous (D2O) solution at 218 K. The sample was prepared by adding 15 mg DNA to 1 cm3 of D2O.
Figure A4 Simulation of the infrared O-H absorption band of HOD in frozen aqueous (D2O) solution at 218 K. The sample was prepared by adding 20 mg DNA to 1 cm3 of D2O.
Figure A5 Simulation of the infrared O-H absorption band of HOD in frozen aqueous (D2O) solution at 218 K. The sample was prepared by adding 50 mg DNA to 1 cm3 of D2O.
2.6 References


Chapter 3

Effects Of Added LiCl And NaCl On DNA Radiation Target Size In Frozen Aqueous Solution
Effects Of Added LiCl And NaCl On DNA Radiation Target Size In Frozen Aqueous Solution.

This chapter reports details of phase separation in frozen aqueous solutions containing salt and DNA. Two phases are formed, called ice-like water and glassy water. Ice-like water is formed from bulk water and glassy water describes water which is perturbed from the normal ice structure by interaction with DNA. The effects of added NaCl and added LiCl on the formation of glassy water are examined. Results are discussed in terms of targets for DNA radiation damage. The concept of target size for the interaction of ionising radiation with DNA in frozen aqueous solutions is used in the analysis, the target for radiation damage being solvated DNA.

3.1 Introduction

DNA is an important target for radiation damage in living organisms. Mechanisms of damage to living cells caused by ionising radiation generally implicate DNA. Studies into radiation-induced DNA damage have examined the effects of a number of salts on the yield of double and single strand breaks [1,2]. Electron spin resonance (ESR) spectroscopy has probed the initial damage process [3,4].

At 77 K an irradiated frozen aqueous solution containing DNA, in the absence of added cosolutes, has an ESR spectrum comprising approximately equal contributions of singlet and doublet features and also resonances due to hydroxy radicals trapped in ice. The doublet is assigned to pyrimidine electron gain centre(s), DNA*; and the singlet is assigned to electron loss centre(s), DNA*+.

Radiolysis products of water in the ice-like phase (reactions 1 to 5) do not interact with DNA and at 77 K result in the formation of trapped hydroxyl radicals and molecular hydrogen.
Radical centres located on DNA are formed directly by interaction of ionising radiation with DNA (reactions 6 and 7) and also indirectly by transfer of electrons and electron holes from the glassy water phase surrounding DNA (reactions 8 to 10).

Addition of NaCl or LiCl to aqueous solutions of DNA increased the number of DNA radicals formed on irradiation of the frozen system [3,4]. DNA samples were prepared by adding aqueous solutions containing LiCl(aq) or NaCl(aq) to a known mass of DNA. The DNA radical concentration (at 130 K) for samples containing 50 mg DNA dissolved in 1 cm$^3$ of salt solution having a concentration of 1 mol dm$^{-3}$ was approximately twice that for DNA solutions with no added salt: figure 3.1. Added salt enhanced the yield of DNA$^-$ more than DNA$^{+\cdot}$. The ESR spectra for systems with a high LiCl or NaCl concentration have significantly more doublet character. For salt concentrations greater than about 0.5 mol dm$^{-3}$ ESR spectra showed features assigned to Cl$_2$$^-\cdot$ and ClOH$^-$ radicals solvated in glassy water.
Figure 3.1 Total DNA Radical Yield In Frozen Aqueous LiCl Solution & NaCl Solution, Data From Malone [4].

- Frozen aqueous LiCl solution
- Frozen aqueous NaCl solution

DNA Radical Yield (Arbitrary Units)

[Salt] / mol dm⁻³

61
Malone [4] explained these results by considering the fate of electrons and electron holes generated upon ionisation of the glassy water surrounding DNA. Electrons are trapped on DNA as there are no other chemical traps in the glassy water phase. Consequently the concentration of DNA•+ increases with increasing amounts of LiCl or NaCl. Electron holes (H
\textsubscript{2}O•+ and Cl• electron loss centres) generated in the glassy phase are converted into ClOH• and Cl\textsubscript{2}•+ radicals which were shown to be trapped at low temperatures and are probably formed by reactions 11 to 16, listed below. Thus hole trapping by formation of ClOH• and Cl\textsubscript{2}•+ competes with trapping by DNA•+, and hence at LiCl concentrations of 6 mol dm\textsuperscript{-3} the electron gain centre DNA•+ accounts for over 90% of the total DNA radical yield.

\begin{align*}
\text{Cl}^- + \gamma & \rightarrow \text{Cl}^* + e^- & 11 \\
\text{H}_2\text{O}^{•+} + \text{Cl}^- & \rightarrow \text{Cl}^* + \text{H}_2\text{O} & 12 \\
\text{Cl}^* + \text{Cl}^- & \rightarrow \text{Cl}_2^{••} & 13 \\
\text{H}_2\text{O}^{•+} + \text{H}_2\text{O} & \rightarrow \text{•OH} + \text{H}_3\text{O}^+ & 14 \\
\text{•OH} + \text{Cl}^- & \rightarrow \text{ClOH}^{••} & 15 \\
\text{ClOH}^{••} + \text{Cl}^- & \leftrightarrow \text{Cl}_2^{••} + \text{OH}^- & 16 
\end{align*}

In contrast to the ESR spectra, frozen aqueous DNA samples which contain NaCl or LiCl do not show a large increase in DNA damage. Gel electrophoresis of plasmid supercoiled DNA, irradiated at 77 K in aqueous solutions, indicates that NaCl and LiCl have relatively little effect on the amounts of DNA single strand breaks (open circular form DNA) or double strand breaks (linear form DNA) [1,2].

This chapter discusses the effect of added NaCl and added LiCl on the hydration of DNA in frozen aqueous solutions. The aim of this research was to probe the effects of salt on the DNA radiation target.
in order to offer an explanation for the increase in total DNA radical yield as detected by ESR spectroscopy in studies of initial DNA radiation damage [3,4]. The concept explored was that DNA solutions which contain either LiCl or NaCl undergo phase separation on freezing. The following model was suggested: hydrated salts phase separate in the DNA system. As the concentration of either LiCl or NaCl salt is increased the amount of ice-like water decreases and the amount of glassy, solvating, water increases. The products of radiation damage to ice-like water are trapped whereas the products of radiation damage to glassy water and chloride ions are not trapped efficiently in the glassy water phase and may migrate to DNA. If added salts increase the amount of glassy water in the vicinity of DNA then the glassy water phase, which is the target for DNA damage, is enlarged. The overall effect is an increase in the total target for DNA damage by gamma irradiation.

3.2 Previous Work

The effect of radiation on frozen aqueous DNA and salt solutions has not been previously reported in the literature. However infrared studies have probed the effects of electrolytes on the secondary structure of DNA. The effect of water on the radical yield of irradiated (frozen) DNA and on the products of irradiated DNA have also been studied and the results are reported below.

3.2.1 DNA Conformations

The conformational structure adopted by DNA depends on the water and salt content of the medium surrounding the nucleic acid [5-10]. At relative humidities of less than 66%, fibres of the Li DNA salt undergo a transition in secondary structure from the B to the C form [5]. With NaDNA fibres a transition occurs from the B form to the A form as the relative humidity decreases. The humidity at which the transition takes place depends on the ion content of the hydrated fibres [6].

Falk and co-workers used infrared and ultra-violet spectroscopy to study oriented DNA films maintained at relative humidities between 0 - 92% [7]. Plots of the dichroic ratio of the DNA infrared absorption band at 1600 cm\(^{-1}\) and of an ultra violet absorption at
260 nm against relative humidity showed a transition between DNA conformations. This structural transition from the C to the B form occurs in the range 55 - 75% relative humidity. The B conformation was found to be the dominant form of fully hydrated DNA.

Hanlon et al examined structural transitions of DNA in aqueous salt solutions [8,9]. The circular dichroism was reported of calf thymus DNA in aqueous solutions of NaCl, KCl, LiCl, CsCl and NH₄Cl at pH 7 and 300 K. Previously published spectra of the A, B and the C forms of DNA were used in the data analysis [10]. Accurate reference spectra of these DNA forms in aqueous solution were calculated using these data, as were the fractional distributions of A, B and C conformations of DNA in salt solutions. In all salt solutions studied, calf thymus DNA underwent a structural transition from the B to the C form with increasing salt concentration. For solutions containing high concentrations of LiCl, CsCl or NH₄Cl a small amount of the A form of DNA was also present in solution. Figures 3.2 and 3.3 show the fractional distributions of the A, B and C conformations of DNA in NaCl and LiCl solutions as calculated by Hanlon et al.

3.2.2 Irradiation Of DNA And The Effects Of Hydration

Schwartz et al [11] irradiated DNA samples held at different humidities at room temperature. The release of unaltered DNA bases was used to monitor DNA damage. The yield of radiation induced free bases was nearly constant between 5 and 30 water molecules per base pair of DNA when irradiated under nitrogen. In the same hydration range the yield decreased when DNA samples were irradiated under oxygen. Above 30 water molecules per DNA base pair, the yield of free bases following irradiation increased rapidly under both nitrogen and oxygen.

The results were accounted for using a model which takes two factors into account: (i) the damage resulting from direct ionisation of DNA when a mixture of the A and B conformers in vacuum dried DNA changes to predominantly the B conformer in fully hydrated DNA; and (ii) the damage resulting from irradiation of DNA hydration water. Two types of water molecules were assumed to be present: type one water was assigned as being close to DNA and type two...
Figure 3.2  Conformations of DNA in NaCl(aq) solutions [8].
Figure 3.3 Conformations of DNA in LiCl(aq) solutions [8].
water was deemed to be further away from DNA. The number of type one water molecules per DNA base pair was used as a parameter to account for the observed patterns.

Release of DNA bases originating from irradiation of type one water occurs predominantly by charge transfer from the direct ionisation of 24 - 30 water molecules per DNA base pair and by attack of hydroxyl radicals generated in the outer, more loosely bound, type two water molecules. In forming DNA lesions resulting in the release of an unaltered base, irradiation of the outer water molecules (solvation number greater than 30 water molecules per DNA base pair) was more efficient than irradiation of the inner water molecules (less than approximately 30 water molecules per DNA base pair) by a factor of approximately 3.3.

The DNA radiation target for direct radiation damage in liquid solutions is DNA plus approximately 30 water molecules per DNA base pair. Irradiation of water in a system with excess of 30 water molecules per DNA base pair produces hydroxyl radicals. In liquid water these radicals may attack DNA. However in frozen aqueous solution these hydroxyl radicals are trapped in ice-like water.

**ESR Studies Of DNA Frozen Aqueous Solutions**

In one of the earliest studies, Gregoli et al examined frozen aqueous solutions of DNA [12]. Samples at four DNA concentrations: 10, 50, 100 and 200 mg cm$^{-3}$ were investigated. These samples were first irradiated at 77 K, annealed to 130 K to eliminate OH$^*$, and re-cooled to 77 K prior to recording ESR spectra. The yield of DNA radicals in frozen aqueous solutions was approximately twice that in dry DNA; absolute yields were not determined. At 77 K both DNA and water radicals contributed to the total radical yield. In samples annealed to 135 K, water radicals were lost and the total radical yield corresponded to DNA located radical centres (Figure 3.4). Radiation energy absorbed in the solvation shell transferred to the DNA via mobile holes and dry (unsolvated) electrons. Water in excess of DNA solvation water was regarded as a separate ice-like phase in which radicals were unable to interact with DNA.
Figure 3.4 The hatch columns represent the \((\text{H}_2\text{O})^+\) radical yield at 77 K. This yield is the same for pure \(\text{H}_2\text{O}\) and for variously concentrated DNA solutions. At 135 K the plot of \((\text{DNA})^+\) yield against DNA concentration is a straight line passing through the origin (curve b). For dry DNA samples, obtained by freeze-drying the variously concentrated DNA solutions, the relationship is also described by a straight line (curve c), but the slope is half that of curve b. Gregoli and co-workers [12].
Huttermann et al studied the influence of hydration water on radicals produced in irradiated DNA [13]. DNA samples were equilibrated for three weeks at relative water humidities of 5%, 32%, 45%, 66%, 76% and 95% (equivalent to approximately 7, 10, 12, 16, 22 and 55 water molecules per DNA base pair respectively). Samples were irradiated at 77 K. The concentration of DNA radicals increased rapidly on going from samples with 7 to those with 22 water molecules per DNA base pair. The radical yield at 55 water molecules per DNA base pair was approximately 75% that found in samples with 22 water molecules per DNA base pair (Figure 3.5). It was concluded that water of hydration is an integral part of the DNA target up to at least 76% relative humidity (ca. 22 water molecules per DNA base pair). However, at 95% relative humidity (ca. 55 water molecules per DNA base pair) the radiation chemistry is a combination of the separate radiation induced processes that occur in ice and in hydrated DNA. The increase in DNA total radical yield at 77 K was attributed to an increase in the target size, the target being DNA and hydration water.

Hutterman and co-workers also noted that changes in the secondary structure of DNA influenced the formation of radical centres produced in the macromolecule [13]. Components of ESR spectra for samples prepared at 66% relative humidity were identical with those obtained from oriented DNA in the A-form. At 66% relative humidity the secondary structure of DNA is intermediate between an irregular dry form and regular A- or B-form. Results for systems prepared at 66% relative humidity appear to 'represent a singularity in behaviour' attributable to this transition in the secondary structure of DNA.

Mroczka and Bernhard studied hydration effects on radical yields in DNA irradiated at 4 K [14]. DNA samples at various levels of hydration were irradiated and their ESR spectra recorded. OH⁺ and H⁺ radicals were not detected in samples with up to approximately 60 water molecules per DNA base pair but these radicals were observed in those samples with a water content of approximately 80 water molecules per DNA base pair or above. This composition corresponds to roughly twice the number of water molecules required to fully hydrate DNA. The absence of radicals derived from
Figure 3.5 Total DNA radical yield at 77 K. Radical yield is normalised to mass of specimen and is shown in relation to hydration water of DNA. Hutterman and co-workers [13].
ice was attributed to damage transfer from water of solvation to DNA and also differential recombination in which hydration water was postulated to be less efficient than bulk water at trapping radicals.

Sevilla and co-workers [15], in an ESR investigation of the effects of hydration water on the absolute yields of radicals in gamma irradiated DNA at 77 K, found that the total DNA radical yield increased four fold on addition of 40 water molecules per DNA base pair. Above this level of hydration, excess water forms a separate, and apparently radiologically independent, ice-like phase. Formation of this ice-like phase was found to remove ca. 10 water molecules per DNA base pair from the hydration water, causing a proportionate drop in the yield of total DNA radical centres. Hydroxyl radicals (OH•) were not detected in DNA hydration water, but were detected in the ice-like phase. Water of hydration was shown to be critical to radical formation in DNA; ice-like water did not contribute to radiation induced DNA damage. Sevilla and co-workers calculated G values (the number of DNA radicals produced per unit energy deposited in the sample expressed in units of μmol J⁻¹) for the total radical yield of DNA at 77 K. Values of G were normalised to the entire sample mass; G(sample). The concept of a target mass (TM) of DNA and glassy water was introduced, and this was used to calculate G normalised to the target mass, G(TM).

\[
G(\text{sample}) = \frac{\text{DNA spins per gram sample}}{\text{energy absorbed per gram sample}}
\]

\[
G(\text{TM}) = \frac{\text{DNA spins per gram TM}}{\text{energy absorbed per gram TM}}
\]

G(sample) and G(TM) plotted as a function of water molecules per DNA base pair reflect the radiation processes taking place in the frozen system. As DNA samples contain progressively more water both G(sample) and G(TM) increase rapidly at the same rate, reaching a maximum at approximately 40 water molecules per DNA base pair (figure 3.6 a and b). Above 40 water molecules per DNA base pair G(sample) rapidly decreases because ice-like water is formed and the sample mass increases, however radiation damage in
Figure 3.6  G values for the total radical yield of DNA(aq) irradiated at 77 K and based on fits to dose-response data at doses < 50 kGy and plotted as a function of DNA hydration (molecules of D$_2$O per DNA base pair) [15]. G(sample) values are normalised to the entire sample mass; plot 3.6a. Values of G(TM) are normalised to the DNA target mass which is the mass of DNA and glassy DNA solvating water; plot 3.6b.

Figure 3.6a

Figure 3.6b
this phase does not migrate to DNA(aq). In contrast G(TM) decreases slightly a little above 40 water molecules per DNA base pair but thereafter maintains a constant value because the target mass remains constant; glassy water is not formed.

In a more recent study of gamma irradiated DNA [16], Sevilla and co-workers showed that hydroxyl radicals were produced in low yield in the glassy water associated with DNA in samples containing 30 - 40 water molecules per DNA base pair. ESR spectra of hydrated DNA samples at 90 K were shown to have a broad low field spectral component almost identical to that of hydroxyl radicals formed by irradiation of 6 mol dm\(^{-3}\) BeF\(_2\)(aq) glass. In DNA samples much of the hydroxyl radical spectrum is obscured due to strong resonances from radicals centred on DNA. An estimated 70% of the electron loss centres (H\(_2\)O\(^+\)) which do not undergo recombination with electrons in the glassy DNA water transfer as 'dry charge' to the DNA; the remaining 30% react with water to produce trapped hydroxyl radicals.

3.3 Experimental

3.3.1 Materials

Highly polymerised sodium salt calf thymus DNA was obtained from BDH Chemicals Limited. DNA of a single batch number was used throughout each experiment. Deuterium oxide (D\(_2\)O 99.9% D) was used as obtained from Goss Limited. Analytical grade sodium chloride and lithium chloride were obtained from Aldrich Chemical Company.

3.3.2 Procedure

As purchased, DNA contains excess sodium chloride. Prior to sample preparation this sodium chloride was removed by dialysis. Typically 25 cm\(^3\) of water was added to 0.5 g of DNA. The resulting solution was left to fully hydrate for at least 24 hours at 277 K before being transferred, by syringe, into cellulose dialysis tubing. The tubing was
sealed and, in early experiments, dialysed against sodium chloride solutions in order of decreasing sodium chloride concentration. However, dialysis of DNA against water for at least 24 hours at 277 K gave DNA of at least the same quality as that obtained by dialysis against several salt solutions of decreasing concentration. This latter method was adopted as the general procedure. DNA was recovered from solution by freeze drying and stored in sealed sample tubes at 277 K until required.

DNA samples were prepared by addition of a known weight of 'NaCl free' NaDNA to water (D\textsubscript{2}O), or NaCl / LiCl salt solutions as appropriate. These samples were left to fully hydrate for at least 24 hours at 277 K before use.

3.3.3 Infrared Spectroscopy

Infrared spectra were recorded using a Perkin Elmer 681 double beam spectrometer. The sample temperature was controlled using a vacuum jacket cell holder. Infrared spectra were recorded at 218 K, but were first cooled beyond this, to about 208 K, using liquid nitrogen. This coolant was then replaced by a dry ice/methanol bath. The temperature of the sample was brought up slowly to 218 K under thermostatic control.

Infrared spectra were simulated using a spread sheet computer package (Microsoft Excel). Simulations were produced by generating curves centred at various wavenumbers, each one representing an infrared absorption. These curves were summed to simulate the actual infrared absorption envelope in the O-H stretching region of isotopically dilute HOD. Simulated spectra were plotted over infrared spectra in the O-H stretching region of HOD in DNA/D\textsubscript{2}O ice. In each sample the relative amount of ice-like HOD and glassy HOD in solution could be determined as a fraction of total HOD present by calculating the area beneath the simulated infrared absorption due to ice-like HOD or glassy HOD, as appropriate, and dividing each area by the area beneath the total O-H absorption envelope. The amount of glassy or ice-like water was calculated assuming that the ratio of glassy HOD to ice-like HOD was equal to the ratio glassy water (glassy HOD + glassy D\textsubscript{2}O) to ice-like water (ice-like HOD + ice-like D\textsubscript{2}O).
3.4 Results

3.4.1 Infrared Studies Of Frozen Aqueous DNA And LiCl Solutions

Frozen aqueous solutions of DNA which contain increasing amounts of LiCl broaden the O-H absorption band of HOD in D$_2$O ice; figure 3.7 and table 3.1. Figure 3.8 shows several infrared spectra of frozen aqueous DNA containing a range of LiCl concentrations (10 mg DNA + 1 cm$^3$ of LiCl(aq) solution). Simulations of these spectra indicated that broadening is due to an increase in the amount of 'glassy' water produced by added LiCl.

Spectra of DNA in frozen aqueous LiCl solutions were simulated by combining a Gaussian band and the infrared O-H absorption band for a frozen solution of DNA in D$_2$O in the absence of LiCl. The infrared O-H absorption in the absence of LiCl represents the contribution from the spectra of both ice-like water and glassy DNA solvation water; the Gaussian band represents the contribution from glassy 'LiCl water'. These two bands are summed together and the resulting band shape fitted to the actual spectrum by eye. The parameters for the Gaussian band were determined from spectra of HOD/D$_2$O in a frozen 10 mol dm$^{-3}$ LiCl glass, figure 3.9.

A computer spreadsheet was used to produce the simulations. A best fit to DNA/LiCl spectra was achieved using a Gaussian band centred at 3360 cm$^{-1}$ with a full width at half height (FWHH) of 244 cm$^{-1}$. The Gaussian band and the O-H absorption band in D$_2$O were scaled to fit the actual spectrum independently until their sum matched that of the infrared spectrum under investigation. Simulation bands and their associated spectra could then be integrated. The simulated and observed spectra are compared in appendix A. The areas beneath the O-H stretch absorption spectra and the best fit simulation bands are summarised in table 3.2.
Figure 3.7 Full Width At Half Height (FWHH) of the O-H stretching mode of HOD in DNA + LiCl solutions and DNA + NaCl solutions at 218 K
Table 3.1 The full width at half height (FWHH) of the O-H infrared absorption band observed for samples of frozen aqueous DNA and LiCl solutions (10 mg DNA + 1 cm³ salt solution).

<table>
<thead>
<tr>
<th>LiCl concentration / mol dm⁻³</th>
<th>Ratio of LiCl to DNA base pairs (Li)/(DNA)</th>
<th>O-H stretch of HOD FWHH ± 2 cm⁻¹ / cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>42</td>
</tr>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>40</td>
</tr>
<tr>
<td>0.10</td>
<td>6.62</td>
<td>41</td>
</tr>
<tr>
<td>0.20</td>
<td>13.24</td>
<td>43</td>
</tr>
<tr>
<td>0.30</td>
<td>19.86</td>
<td>43</td>
</tr>
<tr>
<td>0.40</td>
<td>26.49</td>
<td>46</td>
</tr>
<tr>
<td>0.60</td>
<td>39.73</td>
<td>47</td>
</tr>
<tr>
<td>0.80</td>
<td>52.97</td>
<td>52</td>
</tr>
<tr>
<td>1.00</td>
<td>66.22</td>
<td>52</td>
</tr>
</tbody>
</table>

Table 3.2 Integration of the observed 'real' O-H stretching infrared absorption band and also of the best fit simulation bands from 3550 cm⁻¹ to 3150 cm⁻¹ in frozen DNA and LiCl solutions.

<table>
<thead>
<tr>
<th>[LiCl] / mol dm⁻³</th>
<th>Integral of the 10 mg DNA +1 cm⁻³ D₂O spectrum, scaled to fit spectra / cm⁻¹</th>
<th>Integral of Gaussian band / cm⁻¹</th>
<th>Integral of 'real' spectrum / cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>2776</td>
<td>120</td>
<td>3109</td>
</tr>
<tr>
<td>0.3</td>
<td>2972</td>
<td>383</td>
<td>3587</td>
</tr>
<tr>
<td>0.4</td>
<td>3241</td>
<td>622</td>
<td>3907</td>
</tr>
<tr>
<td>0.6</td>
<td>3339</td>
<td>766</td>
<td>4294</td>
</tr>
<tr>
<td>0.8</td>
<td>4197</td>
<td>1915</td>
<td>6675</td>
</tr>
<tr>
<td>1.0</td>
<td>4038</td>
<td>2155</td>
<td>6489</td>
</tr>
</tbody>
</table>
Figure 3.8 Infrared spectra of the O-H stretching band of HOD in frozen aqueous (D2O) solutions prepared using DNA and LiCl(aq) (10 mg DNA + 1 cm$^3$ of LiCl solution).
Figure 3.9 The infrared O-H stretching band of isotopically dilute HOD in 10 mol dm$^{-3}$ LiCl / D$_2$O in frozen aqueous solution at 218 K

![Graph showing the infrared O-H stretching band of isotopically dilute HOD in 10 mol dm$^{-3}$ LiCl / D$_2$O in frozen aqueous solution at 218 K. The graph plots absorbance against wavenumber in cm$^{-1}$, with a peak at around 3300 cm$^{-1}$ with absorbance values reaching up to 120.]}
The area beneath an absorption band is proportional to the number of oscillators. Dividing the area beneath the Gaussian simulation band \( (A_{\text{Gaussian}}) \) by the total area beneath the O-H absorption band of the 'real' spectrum \( (A_{\text{real}}) \) yields the amount of glassy HOD water molecules, formed by addition of LiCl, expressed as a percentage of total HOD water molecules in both glassy and ice-like water. This percentage equals the fraction of glassy D\textsubscript{2}O water in solution, assuming that HOD does not preferentially hydrate either DNA or LiCl. This calculation assumed that the extinction coefficients of O-H oscillators in ice-like water and in glassy water are the same. This assumption is supported by the simulated spectra which showed that a loss of intensity in the ice-like water was directly equivalent to a gain in intensity of the infrared O-H stretching band associated with glassy 'LiCl water'.

The fractions of glassy water at different LiCl concentrations are recorded in table 3.3 and illustrated in figure 3.10. The gradient of the plot in figure 3.10 is approximately 3x10\(^{-4}\) mol cm\(^{-3}\) water molecules per unit increase in the ratio of moles LiCl per mole DNA base pair contained in 1 cm\(^3\) of solution at room temperature. In this system, containing 1.51x10\(^{-5}\) moles of DNA base pairs, this gradient is equivalent to 19 moles of water per mole of LiCl. In order to test the validity of simulated spectra, figure 3.10 and table 3.3 include data calculated by dividing \( A_{\text{Gaussian}} \) by the total area beneath the simulated band envelope of the O-H stretching mode \( (A_{\text{simulation}}) \). The percentage increase in glassy water calculated by \( A_{\text{Gaussian}}/A_{\text{real}} \) is slightly less than that calculated from \( A_{\text{Gaussian}}/A_{\text{simulation}} \) because \( A_{\text{real}} \) is generally greater than \( A_{\text{simulation}} \). This pattern is especially evident at high LiCl concentrations where there is a slight increase in the width of the Lorentzian band produced by the ice-like water. This difference amounts to a few percent at high LiCl concentrations and does not affect the general conclusion that increasing amounts of glassy water are formed in frozen aqueous solutions of DNA which contain increasing amounts of LiCl.
Table 3.3 Increase in glassy water with increasing amounts of LiCl in frozen aqueous solutions containing 10 mg DNA (1.51 \times 10^{-6} \text{ mole DNA base pairs}).

<table>
<thead>
<tr>
<th>Ratio [moles LiCl]/[moles DNA base pairs]</th>
<th>Percent of &quot;LiCl glassy water&quot; molecules given by $A_{\text{Gaussian}}/A_{\text{simulation}}$ (As percentage of total water)</th>
<th>Percent of &quot;LiCl glassy water&quot; molecules given by $A_{\text{Gaussian}}/A_{\text{real}}$ (As percentage of total water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.62 (0.1 mol dm$^{-3}$)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>19.86 (0.3 mol dm$^{-3}$)</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>26.49 (0.4 mol dm$^{-3}$)</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>39.73 (0.6 mol dm$^{-3}$)</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>52.97 (0.8 mol dm$^{-3}$)</td>
<td>31</td>
<td>29</td>
</tr>
<tr>
<td>66.22 (1.0 mol dm$^{-3}$)</td>
<td>35</td>
<td>33</td>
</tr>
</tbody>
</table>
Figure 3.10 Amount of glassy water associated with LiCl and DNA in frozen aqueous solutions containing 10 mg DNA and 1 cm³ of LiCl(aq) solution

- moles of Water (A_Gaussian/A_real)
- moles water (A_Gaussian/A_simulation)

Slope = 2.9E-4 (i.e. 19 moles water per mol LiCl added to 10 mg DNA)
Infrared Studies Of Frozen Aqueous DNA And NaCl Solutions

In samples of frozen aqueous solutions containing DNA and NaCl the full width at half height (FWHH) of the O-H infrared stretching band centred at 3300 cm\(^{-1}\) is not affected by increasing amounts of NaCl in the sample (figure 3.7). Addition of NaCl produces an O-H stretching infrared band centred near 3440 cm\(^{-1}\). This absorption band is characteristic of NaCl hydrate, NaCl\(\cdot\)2H\(_2\)O [17]. Figure 3.11 summarises infrared spectra of frozen solutions containing 10 mg DNA and NaCl(aq) between 0 and 0.8 mol dm\(^{-3}\). The band assigned to NaCl hydrate becomes apparent for solutions having concentrations of NaCl greater than 0.4 mol dm\(^{-3}\).

Infrared spectra were simulated in a similar fashion to the procedures described for spectra of solutions containing LiCl. The results are shown in appendix B. A Lorentzian curve with a FWHH equal to 24 cm\(^{-1}\), centred at 3440 cm\(^{-1}\), was used to model the O-H stretching mode of HOD which was assigned to the NaCl hydrate band. A Gaussian curve gave a better fit for spectra of solutions with low NaCl concentrations and a Lorentzian curve gave the best fit for solutions with higher NaCl concentrations. To maintain a constant band shape for the simulation across the range of NaCl concentrations a Lorentzian curve provided the best overall band shape.

The remaining O-H absorption band centred at 3300 cm\(^{-1}\) was simulated, as before, by scaling an infrared spectrum of the O-H stretching mode of isotopically dilute HOD in a solution prepared using 10 mg DNA + 1 cm\(^3\) D\(_2\)O. This simulation band represents the infrared absorption due to the ice-like water and the glassy DNA solvating water. The simulated band for NaCl hydrate was integrated yielding the area under the peak (\(A_{\text{hydrate}}\)). Similarly the recorded spectrum was integrated between 3550 cm\(^{-1}\) and 3200 cm\(^{-1}\) yielding the area beneath the O-H absorption band envelope (\(A_{\text{spectrum}}\)); table 3.4. Figure 3.12 and table 3.5 present data for \(A_{\text{hydrate}}/A_{\text{spectrum}}\) with increasing NaCl concentration. For comparison with data calculated by the ratio \(A_{\text{hydrate}}/A_{\text{simulation}}\), the area beneath the simulated O-H infrared absorption band envelope (\(A_{\text{simulation}}\)) was calculated and
Figure 3.11 Infrared spectra of the O-H stretching mode of HOD in frozen aqueous (D2O) solutions of DNA containing NaCl (10 mg DNA in 1 cm3 of salt solution).
Table 3.4 Integration from 3500cm⁻¹ to 3200cm⁻¹ of the O-H stretching absorption and the simulation bands used to model O-H stretching modes in frozen DNA/NaCl solutions.

<table>
<thead>
<tr>
<th>[NaCl] / mol dm⁻³</th>
<th>Area Beneath Simulation Spectrum, ( A_{\text{simulation}} ) / cm⁻¹</th>
<th>Area Beneath Lorentzian (simulated NaCl hydrate) Band, ( A_{\text{hydrate}} ) / cm⁻¹</th>
<th>Area Beneath Spectrum, ( A_{\text{spectrum}} ) / cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>1957</td>
<td>10</td>
<td>1972</td>
</tr>
<tr>
<td>0.2</td>
<td>3261</td>
<td>10</td>
<td>3210</td>
</tr>
<tr>
<td>0.3</td>
<td>3247</td>
<td>10</td>
<td>3178</td>
</tr>
<tr>
<td>0.4</td>
<td>4025</td>
<td>34</td>
<td>4150</td>
</tr>
<tr>
<td>0.5</td>
<td>5204</td>
<td>69</td>
<td>5264</td>
</tr>
<tr>
<td>0.6</td>
<td>4272</td>
<td>62</td>
<td>4277</td>
</tr>
<tr>
<td>0.7</td>
<td>4433</td>
<td>77</td>
<td>4655</td>
</tr>
<tr>
<td>0.8</td>
<td>3413</td>
<td>86</td>
<td>3675</td>
</tr>
</tbody>
</table>

* \( A_{\text{simulation}} \) is the area beneath the simulated infrared absorption envelope obtained by summing the area beneath the O-H stretching mode in 10mg DNA +1 cm⁻³ D₂O samples and the area beneath the Lorentzian band which simulates the O-H stretching mode of HOD assigned to the NaCl hydrate band. Both these bands were scaled to fit the observed infrared spectra.
Figure 3.12 Amount of water associated with NaCl in solutions prepared by adding 1 cm3 of salt solution to 10mg DNA and freezing to 218 K

- A hydrate/A simulation = moles of water calculated by dividing the area beneath the Lorentzian band (band E in appendix B, figures B1 - B8) by the area beneath the O-H stretching region of HOD (spectrum labeled B in appendix B, figures B1 - B8).

- A hydrate/A spectrum = moles of water calculated by dividing the area beneath the Lorentzian band (band E in appendix B, figures B1 - B8) by the area beneath the simulated O-H stretching band envelope (band A in appendix B, figures B1 - B8).

- Linear regression of data having a NaCl(aq) concentration greater than 3.00E-4 mol cm-3.
  1. Slope = 2.0, Intercept = -4E-4
  2. Curve fit to data
Table 3.5 Glassy water associated with NaCl hydrate in frozen aqueous solutions containing 10 mg DNA (relative molecular mass calf thymus DNA = 662.15 g / mol base pairs).

<table>
<thead>
<tr>
<th>Ratio [moles NaCl] (moles DNA base pairs].</th>
<th>Molarity of NaCl solution / mol dm⁻³ is given in brackets</th>
<th>Moles of additional 'NaCl glassy water' molecules given by ( \Delta h_{\text{hydrate}}/\Delta h_{\text{simulation}} )</th>
<th>Moles Of Additional NaCl Glassy Water Molecules Given By ( \Delta h_{\text{hydrate}}/\Delta h_{\text{experiment}} )</th>
<th>Percentage &quot;NaCl glassy water&quot; is given in brackets</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.62 (0.1)</td>
<td>&lt; 3x10⁻⁴ (&lt; 0.5)</td>
<td>&lt; 3x10⁻⁴ (&lt; 0.5)</td>
<td>&lt; 3x10⁻⁴ (&lt; 0.5)</td>
<td></td>
</tr>
<tr>
<td>13.24 (0.2)</td>
<td>&lt; 3x10⁻⁴ (&lt; 0.5)</td>
<td>&lt; 3x10⁻⁴ (&lt; 0.5)</td>
<td>&lt; 3x10⁻⁴ (&lt; 0.5)</td>
<td></td>
</tr>
<tr>
<td>19.86 (0.3)</td>
<td>&lt; 3x10⁻⁴ (&lt; 0.5)</td>
<td>&lt; 3x10⁻⁴ (&lt; 0.5)</td>
<td>&lt; 3x10⁻⁴ (&lt; 0.5)</td>
<td></td>
</tr>
<tr>
<td>26.49 (0.4)</td>
<td>4x10⁻⁴ (0.8)</td>
<td>4x10⁻⁴ (0.8)</td>
<td>4x10⁻⁴ (0.8)</td>
<td></td>
</tr>
<tr>
<td>33.11 (0.5)</td>
<td>7x10⁻⁴ (1.3)</td>
<td>7x10⁻⁴ (1.3)</td>
<td>7x10⁻⁴ (1.3)</td>
<td></td>
</tr>
<tr>
<td>39.73 (0.6)</td>
<td>8x10⁻⁴ (1.5)</td>
<td>8x10⁻⁴ (1.5)</td>
<td>8x10⁻⁴ (1.5)</td>
<td></td>
</tr>
<tr>
<td>46.35 (0.7)</td>
<td>9x10⁻⁴ (1.7)</td>
<td>9x10⁻⁴ (1.7)</td>
<td>9x10⁻⁴ (1.7)</td>
<td></td>
</tr>
<tr>
<td>52.97 (0.8)</td>
<td>1.4x10⁻³ (2.5)</td>
<td>1.3x10⁻³ (2.3)</td>
<td>1.3x10⁻³ (2.3)</td>
<td></td>
</tr>
</tbody>
</table>
Hydrate/Asimulation data are also included in table 3.5 and figure 3.12. The differences between Hydrate/A spectrum and Hydrate/Asimulation are small. The O-H stretching band attributed to NaCl hydrate was difficult to simulate for spectra of DNA solutions containing less than 0.4 mol dm$^{-3}$ NaCl. The hydrate band has very low intensity in comparison to other O-H bands in the infrared spectra. The percentage 'NaCl glassy water' calculated from infrared spectra of DNA solutions having concentrations of NaCl between 0.1 - 0.3 mol dm$^{-3}$ are best described as containing less than 0.5% "NaCl glassy water".

Linear regression of the data points above 0.3 mol dm$^{-3}$ NaCl (20 NaCl/DNA base pair) in figure 3.12 yields a straight line having a gradient of 2 moles of water per mole NaCl and an intercept of $-4 \times 10^{-4}$ moles water. This result is consistent with a NaCl hydrate phase, NaCl$_2$H$_2$O, being formed in frozen solutions of DNA and NaCl above 0.2 mol dm$^{-3}$ NaCl, i.e. at approximately 13 moles NaCl per mole DNA base pairs. The composition of the NaCl hydrate phase is consistent with the hydrate NaCl$_2$H$_2$O as reported in low temperature infrared spectroscopic studies by Strauss et al [17].

3.4.3 Summary Of Results

Frozen solutions of DNA contain two phases, one consists of DNA plus glassy water and the other is ice-like (bulk) water. On freezing DNA solutions containing LiCl(aq) the salt accumulates in the glassy water DNA phase rather than forming a third phase. The number of glassy water molecules in the DNA phase is increased by approximately $3 \times 10^{-4}$ mol cm$^{-3}$ per unit increase in the ratio of moles of LiCl per mole of DNA base pairs calculated for 1 cm$^3$ of solution at room temperature. The increase in glassy water with increasing amount of LiCl does not depend solely on the amount of LiCl in solution. The increase in glassy water depends on the number of LiCl molecules per DNA base pair in the system.

With DNA solutions containing NaCl(aq) salt accumulates in the glassy DNA phase until the ratio of NaCl/DNA base pairs is approximately 13. Above 13 moles NaCl per mole of DNA base pairs a third phase is formed which consists of the hydrate NaCl$_2$H$_2$O. Formation of the
Salt hydrate results in 2 moles of ice-like water being lost per mole of NaCl in solution. This water forms NaCl hydrate water and the rate of loss is independent of the number of DNA base pairs in solution.

3.5 Discussion

3.5.1 DNA Conformations

Some conformational changes to solvated DNA probably occur when salt is added prior to freezing. The proportions of DNA in the A, B or C forms can be determined using Hanlon's data [8]. Hanlon et al. used solutions prepared by adding 10 mg of DNA to 10 cm$^3$ of salt solution. In the study reported in this thesis 10 mg DNA was added to 1 cm$^3$ of solution. Thus the ratio of salt to DNA base pairs in this work is one tenth of that used by Hanlon et al. In this work the highest salt concentration used was 1 mol dm$^{-3}$ (salt/DNA base pairs = 66.2). In terms of salt per DNA base pair this composition is equivalent to a solution of 10 mg DNA in 10 cm$^3$ of 0.1 mol dm$^{-3}$ salt. At this concentration Hanlon et al. found that DNA is predominantly in the B-form, with about 10% in the C-DNA form in LiCl solution and less than 5% in the C-DNA form in NaCl solution (figures 3.2 and 3.3).

On freezing the solution, DNA(aq) and salt are excluded from the ice-like phase. The resulting localised high salt concentration does not significantly alter the DNA conformation. For example the transition from B-DNA to C-DNA or A-DNA takes place over several hours. The samples used in this study are frozen sufficiently rapidly that no substantial transition from B-DNA occurs.

3.5.2 NaCl Hydrate Phase

The results reported in this study show that a NaCl hydrate phase, NaCl$_2$H$_2$O, is formed in frozen solutions at approximately 13 moles NaCl per mole DNA base pairs or greater. This result is consistent with previous ESR studies [3,4]. In this study DNA solutions were prepared by adding 1 cm$^3$ of NaCl(aq) with a concentration above 0.2 mol dm$^{-3}$ to 10 mg DNA ($1.51 	imes 10^{-5}$ moles of DNA base pairs). Previous ESR studies [3,4] show that ESR spectra of irradiated frozen samples of DNA in solutions containing less than 1 mol dm$^{-3}$ NaCl(aq)
have Cl$_2$O$^-$ features which are relatively broad, suggesting a glassy medium, whereas at NaCl concentrations greater than 1 mol dm$^{-3}$ relatively sharp Cl$_2$O$^-$ features are also detected, suggesting the formation of a crystalline salt hydrate phase. These results are for DNA samples containing 50 mg DNA plus 1 cm$^3$ of salt solution. Thus a NaCl concentration of 1 mol dm$^{-3}$ is equivalent to approximately 13 moles NaCl per mole DNA base pairs.

The infrared study reported here indicates that on freezing solutions with up to approximately 13 moles NaCl per mole of DNA base pairs, NaCl enters the DNA glassy water phase. Thus at low salt concentrations the frozen solution consists of two phases, a bulk ice-like water phase and a glassy water phase, which contains DNA and NaCl. A third phase, NaCl.2H$_2$O, forms when glassy DNA water cannot support further addition of NaCl. At this point ice-like water from bulk solution is scavenged to form salt hydrate.

### 3.5.3 DNA Target In Frozen Aqueous Solutions

The increase in the amount of glassy water and LiCl in frozen aqueous DNA solutions can be used to calculate the corresponding increase in the target for DNA radiation induced damage. This target can also be calculated for DNA solutions which contain NaCl. However with NaCl the assumption has to be made that on freezing the formation of ice-like water excludes both DNA(aq) and NaCl(aq) and that at high salt concentrations the glassy DNA phase and the crystalline NaCl hydrate phase are adjacent; radicals formed in the NaCl hydrate phase are able to migrate to DNA.

The increase in the DNA radiation target brought about by increasing amounts of LiCl or NaCl in solution can be compared with the increase in the DNA radical yield obtained from the ESR study of radiation induced DNA radicals [4].

### I Sodium Chloride And DNA Solutions

Sevilla et al [15] introduced the concept of a target mass for frozen DNA(aq) systems. This target mass is the mass of DNA and water in the glassy water phase. In extending this concept to frozen DNA(aq) solutions which contain NaCl the target mass has been calculated in
two ways. The first, termed $T_{DNAT+water}$, considers the DNA target mass to consist of DNA and target water (water which is able to transfer radiation induced radicals to DNA). This target water is glassy water associated with DNA and the hydrate water associated with the salt. The second target mass calculated is termed $T_{DNAT+water+NaCl}$ and is the mass of DNA, glassy water and the salt hydrate NaCl,$2H_2O$.

TM values were calculated as follows:

\[
T_{DNAT+water} = M(DNA) + M(\text{glassy water}) + M(\text{salt hydrate water})
\]

\[
T_{DNAT+water+NaCl} = M(DNA) + M(\text{glassy water}) + M(\text{NaCl} \cdot 2H_2O)
\]

$M(DNA)$ is the mass of DNA in solution; $M(\text{glassy water})$ is the mass of 30 water molecules per DNA base pair and $M(\text{salt hydrate water})$ is the mass of water in the salt hydrate. $T_{DNAT+water}$ and $T_{DNAT+water+NaCl}$ have been calculated (table 3.6) for frozen solutions of 50 mg DNA in 1 cm$^3$ of NaCl(aq) as used by Malone [4].

Figure 3.13 summarises the total DNA radical yield for various NaCl concentrations observed using ESR [4]. DNA radical yields have been normalised so that the yield in the absence of NaCl(aq) equals one:

\[
\text{Normalised radical yield} = \frac{\text{DNA radical yield at the appropriate } [\text{NaCl}]}{\text{DNA radical yield in } \text{DNA(aq)} \text{ at } [\text{NaCl}] = 0 \text{ mol dm}^{-3}}
\]

In order to compare the increase in DNA radical yield with the increase in $T_{DNAT+water}$ and $T_{DNAT+water+NaCl}$, target masses have been normalised so that TM in the absence of NaCl equals 1.0.

Figure 3.13 includes calculated values of normalised $T_{DNAT+water}$ and normalised $T_{DNAT+water+NaCl}$ for comparison with normalised radical yields. Both TM quantities correlate well with the increase in radical yield. However, normalised $T_{DNAT+water+NaCl}$ shows the best fit to the radical yield data.
Table 3.6  Calculated radiation target mass (TM) values for frozen aqueous solutions prepared by adding 50 mg DNA to 1 cm$^3$ of NaCl(aq) solution.

<table>
<thead>
<tr>
<th>[NaCl] / mol dm$^{-3}$</th>
<th>Mass of DNA and glassy water / g</th>
<th>Mass of hydrate water / g</th>
<th>Mass of salt hydrate (NaCl.2H$_2$O) / g</th>
<th>Mass of target water &amp; DNA (TM$_{DNA}$+water) / g</th>
<th>Mass of target DNA, glassy water &amp; NaCl.2H$<em>2$O (TM$</em>{DNA}$+water+NaCl) / g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0910</td>
<td>0</td>
<td>0</td>
<td>0.091</td>
<td>0.091</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0910</td>
<td>0.0180</td>
<td>0.0472</td>
<td>0.1090</td>
<td>0.1382</td>
</tr>
<tr>
<td>1</td>
<td>0.0910</td>
<td>0.0360</td>
<td>0.0944</td>
<td>0.1270</td>
<td>0.1854</td>
</tr>
<tr>
<td>2</td>
<td>0.0910</td>
<td>0.0720</td>
<td>0.1888</td>
<td>0.1630</td>
<td>0.2798</td>
</tr>
<tr>
<td>3</td>
<td>0.0910</td>
<td>0.1080</td>
<td>0.2832</td>
<td>0.1990</td>
<td>0.3742</td>
</tr>
<tr>
<td>4</td>
<td>0.0910</td>
<td>0.1440</td>
<td>0.3776</td>
<td>0.2350</td>
<td>0.4686</td>
</tr>
<tr>
<td>5</td>
<td>0.0910</td>
<td>0.1800</td>
<td>0.4720</td>
<td>0.2710</td>
<td>0.5630</td>
</tr>
<tr>
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<td>0.1980</td>
<td>0.5192</td>
<td>0.2890</td>
<td>0.6102</td>
</tr>
</tbody>
</table>
Figure 3.13 Comparison of the increase in (A) DNA radical yield with the increase in radiation target mass of (B) DNA, glassy water and salt hydrate water and (C) DNA, NaCl, glassy water and salt hydrate water (radical yield data from [4]).
Normalised target masses were calculated as follows:

\[
\text{Normalised } TM_{\text{DNA+water}} = \frac{M(\text{DNA}) + M(\text{glassy water}) + M(\text{salt hydrate water})}{M(\text{DNA}) + M(\text{glassy water}) \text{ at } [\text{NaCl}] = 0 \text{ mol dm}^{-3}}
\]

\[
\text{Normalised } TM_{\text{DNA+water+NaCl}} = \frac{M(\text{DNA}) + M(\text{glassy water}) + M(\text{NaCl}_2\text{H}_2\text{O})}{M(\text{DNA}) + M(\text{glassy water}) \text{ at } [\text{NaCl}] = 0 \text{ mol dm}^{-3}}
\]

The increase in DNA radical yield predicted by normalised values of \( TM_{\text{DNA+water+NaCl}} \) is slightly greater than that observed (figure 3.13) because not all radicals formed in the DNA target are able to migrate to DNA, for example electron holes (\( \text{H}_2\text{O}^+ \) and \( \text{Cl}^- \)) may be trapped as \( \text{Cl}_2^+ \) and \( \text{ClO}_2^- \) as well as yielding \( \text{DNA}^{++} \). The increase in radical yield predicted by \( TM_{\text{DNA+water}} \) is less than that observed because the target includes NaCl as well as glassy water and salt hydrate water.

On freezing, the NaCl hydrate phase separates in the presence of solvated DNA. If radiation damage to the NaCl hydrate phase is able to migrate to DNA then the formation of \( \text{NaCl}_2\text{H}_2\text{O} \) increases the DNA radiation target by increasing the mass of the radiation target for DNA damage, which is the mass of \( \text{NaCl}_2\text{H}_2\text{O} \), DNA and glassy water associated with DNA. Frozen DNA(aq) solutions containing progressively more NaCl have decreasing amounts of radiologically inert ice-like water and increasing amounts of NaCl and salt hydrate water. Radiation damage in the salt hydrate phase (e.g. electrons and to a lesser extent electron loss centres) migrate to DNA. Hence radiation damage which would, in the absence of NaCl, remain in bulk solution interacts with DNA. The DNA radical yield is therefore greater when NaCl is present in solution because secondary radiation damage which originates in the glassy water phase is enhanced.
II  Lithium Chloride And DNA Solutions

Figure 3.14 summarises the DNA radical yield data observed by ESR of frozen DNA(aq) solutions containing LiCl [4]. These data have been normalised so that the DNA radical yield in the absence of LiCl equals 1.0.

\[
\text{Normalised radical yield} = \frac{\text{DNA radical yield at the appropriate } [\text{LiCl}]}{\text{DNA radical yield in DNA(aq) at } [\text{LiCl}] = 0 \text{ mol dm}^{-3}}
\]

Two target masses have also been calculated: \(T_{\text{DNA+water}}\) is the mass of DNA and glassy water in frozen solution and \(T_{\text{DNA+water+LiCl}}\) is the mass of DNA glassy water and LiCl in solution. The procedure used to calculate \(T_{\text{DNA+water}}\) and \(T_{\text{DNA+water+LiCl}}\) was similar to that used in the previous section for DNA(aq) solutions containing NaCl.

However, as LiCl does not form a third salt hydrate phase, but enters the DNA glassy water phase, the formation of glassy water depends on the ratio of moles of LiCl to moles of DNA base pairs and not solely on LiCl concentration.

TM values were calculated as follows:

\[
T_{\text{DNA+water}} = M(\text{DNA}) + M(\text{glassy water})
\]

\[
T_{\text{DNA+water+LiCl}} = M(\text{DNA}) + M(\text{glassy water}) + M(\text{LiCl})
\]

\(M(\text{DNA})\) is the mass of DNA in solution and \(M(\text{glassy water})\) is the mass of water in the DNA glassy water phase, this includes glassy water produced by LiCl(aq). Values of \(T_{\text{DNA+water}}\) and \(T_{\text{DNA+water+LiCl}}\) have been calculated (table 3.7) for frozen samples prepared by adding 50 mg DNA to 1 cm\(^3\) of LiCl(aq) solution, as used by Malone[4].
Figure 3.14 Comparison of the increase in the DNA radical yield and the increase in target glassy water. Data for both DNA radical yield and moles of glassy water have been normalised so that both equal 1.0 in the absence of LiCl.

- (A) Radical yield data taken from [4] and normalised so that the yield is equal to 1.0 in the absence of LiCl (i.e. at 0 LiCl per DNA base pair).
- (B) Target mass of glassy water and DNA normalised so that this target mass equals 1.0 in the absence of LiCl (i.e. at 0 LiCl per DNA base pair).
- (C) Normalised target mass of DNA and glassy water only (TM DNA + glassy water)
- (D) Target mass of LiCl, glassy water and DNA normalised so that this target mass equals 1.0 in the absence of LiCl (i.e. at 0 LiCl per DNA base pair).
- (E) Normalised target mass (TM DNA + LiCl + glassy water)
Table 3.7 DNA radiation target mass (TM) and normalised TM for frozen aqueous solutions prepared by adding 50 mg DNA to 1 cm³ of LiCl(aq) solution.

<table>
<thead>
<tr>
<th>(1) Molecules of LiCl per DNA base pair LiCl/DNA</th>
<th>(2) Mass of DNA and DNA glassy water (TM\text{DNA} +\text{water})/\text{g}</th>
<th>(3) Values in column 2 normalised i.e. divided by mass of DNA &amp; DNA glassy water</th>
<th>(4) Target Mass of glassy water, DNA and LiCl (TM\text{DNA} +\text{water}+\text{LiCl})/\text{g}</th>
<th>(5) Values in column 4 normalised divided by mass of DNA &amp; DNA glassy water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0908</td>
<td>1.00</td>
<td>0.0908</td>
<td>1.00</td>
</tr>
<tr>
<td>6.62</td>
<td>0.1308</td>
<td>1.44</td>
<td>0.1520</td>
<td>1.67</td>
</tr>
<tr>
<td>19.86</td>
<td>0.2008</td>
<td>2.21</td>
<td>0.2644</td>
<td>2.91</td>
</tr>
<tr>
<td>26.49</td>
<td>0.2508</td>
<td>2.76</td>
<td>0.3356</td>
<td>3.70</td>
</tr>
<tr>
<td>39.73</td>
<td>0.2808</td>
<td>3.09</td>
<td>0.4080</td>
<td>4.49</td>
</tr>
<tr>
<td>52.97</td>
<td>0.4008</td>
<td>4.41</td>
<td>0.5703</td>
<td>6.28</td>
</tr>
<tr>
<td>66.22</td>
<td>0.4408</td>
<td>4.86</td>
<td>0.6528</td>
<td>7.19</td>
</tr>
</tbody>
</table>
As in the previous section both \( \text{TM}_{\text{DNA+water}} \) and \( \text{TM}_{\text{DNA+water+LiCl}} \) target masses have been normalised so that \( \text{TM}_{\text{DNA+water}} \) and \( \text{TM}_{\text{DNA+water+LiCl}} \) in the absence of LiCl, equals 1.0.

\[
\text{Normalised } \text{TM}_{\text{DNA+water}} = \frac{M(\text{DNA}) + M(\text{glassy water}) \text{ at the appropriate } [\text{LiCl}]}{M(\text{DNA}) + M(\text{glassy water}) \text{ at } [\text{LiCl}] = 0 \text{ mol dm}^{-3}}
\]

\[
\text{Normalised } \text{TM}_{\text{DNA+water+LiCl}} = \frac{M(\text{DNA}) + M(\text{glassy water}) + M(\text{LiCl})}{M(\text{DNA}) + M(\text{glassy water}) \text{ at } [\text{LiCl}] = 0 \text{ mol dm}^{-3}}
\]

Figure 3.14 includes normalised \( \text{TM}_{\text{DNA+water}} \) and normalised \( \text{TM}_{\text{DNA+water+LiCl}} \) values for comparison with the normalised DNA radical yield data. Both normalised \( \text{TM}_{\text{DNA+water}} \) and normalised \( \text{TM}_{\text{DNA+water+LiCl}} \) data give a reasonable fit to the radical yield data.

ESR studies show that migration of radical centres from the glassy water phase to DNA is not 100% efficient. In particular electron loss centres (\( \text{H}_2\text{O}^{+*} \) and \( \text{Cl}^{-} \)) do not all migrate to DNA and at high salt concentrations a large proportion of electron loss centres are trapped in the glassy water phase as \( \text{Cl}_2^{2-} \text{(aq)} \) and \( \text{ClOH}^{+}(\text{aq}) \). For this reason, although \( \text{TM}_{\text{DNA+water+LiCl}} \) appears to overestimate radiation-induced DNA damage (figure 3.14), \( \text{TM}_{\text{DNA+water+LiCl}} \) gives a better indication of the DNA target than does \( \text{TM}_{\text{DNA+water}} \). In figure 3.14 the plots of normalised \( \text{TM}_{\text{DNA+water+LiCl}} \) and normalised DNA radical yield would coincide only if all radicals produced in the DNA target migrate to DNA. Although \( \text{TM}_{\text{DNA+water}} \) seems to fit the observed radical yield data in the range 0 to 30 LiCl molecules per DNA base pair, \( \text{TM}_{\text{DNA+water}} \) does not give the best indication of DNA target size because \( \text{TM}_{\text{DNA+water}} \) underestimates the target size above 30 LiCl molecules per DNA base pair.

### 3.6 Conclusions

The increase in the mass of the glassy water phase, brought about by the presence of LiCl (or the formation of a salt hydrate phase with
NaCl present in solution) has been used to account for the increase in DNA radical yield on irradiation of frozen aqueous systems.

Increases in DNA radical yield can be correlated with increases in target mass. In frozen solutions containing NaCl or LiCl the DNA target mass is the mass of DNA plus the mass of glassy water plus the mass of the salt (or salt hydrate if formed). Normalising this mass by dividing by the DNA target mass in the absence of salts (mass of DNA plus the mass of glassy water associated with DNA) yields a factor which is approximately equal to the increase in DNA radical yield. For example, in a frozen DNA solution, prepared by adding 50 mg DNA to 1 cm$^3$ of NaCl(aq) solution at a concentration of 3 mol dm$^{-3}$, the DNA target mass is four times greater than the DNA target mass in the absence of NaCl. The total DNA radical yield observed by ESR spectroscopy is approximately three and a half times greater than the total DNA radical yield in the absence of NaCl [4]. The increase in DNA target mass is greater than the observed increase in radical yield because i. migration of electrons and electron holes onto DNA is not 100% efficient and ii. recombination probably takes place so that a proportion of radical centres formed on DNA are destroyed by electron gain (e.g. DNA$^{+\circ}$ + e$^{-}$ $\rightarrow$ DNA).

In DNA solutions which contain NaCl or LiCl there are chemical and physical processes which prevent a proportion of the radiation induced damage reaching DNA in the target mass. One of these is "hole trapping" whereby electron holes (electron loss centres) at 77 K are converted into Cl$_2$$^{-\circ}$ and ClOH$^{\circ}$ which are trapped in the target mass and do not form DNA$^{+\circ}$.

DNA is the target for radiation induced damage. In dilute fluid solution the majority of ionising events occur to the solvent and the majority of DNA damage is due to the indirect effect of water radicals. In frozen aqueous solutions (and in concentrated solutions of DNA and possibly DNA in cell nuclei) the indirect effect does not dominate. In frozen aqueous solution the target consists of DNA and its glassy water solvating phase of approximately 30 water molecules per DNA base pair. Radiation damage to ice-like water is outside of this target and does not migrate to DNA.
Appendix A

Infrared Spectra of DNA(aq) + LiCl(aq)

Simulated and "real" infrared spectra of frozen aqueous solutions containing DNA and LiCl (figures A1 - A6).
Figure A1  Simulation of the infrared absorption band of the O-H stretching mode of HOD in a sample of 10 mg DNA in 1 cm³ of 0.1 mol dm⁻³ LiCl(aq) at 218 K.

(A) Sum of bands C and D (infrared O-H band of 10 mg DNA + Gaussian band)
(B) Infrared spectrum of 0.1 mol dm⁻³ LiCl(aq) + 10 mg DNA at 218 K
(C) Infrared spectrum of 10 mg DNA(aq) at 218 K (offset by -10)
(D) Gaussian band (Offset by -10)
Figure A2  Simulation of the infrared O-H absorption band of HOD in a sample of 10 mg DNA in 1 cm-3 of 0.3 mol dm-3 LiCl(aq) at 218 K
Figure A3  Simulation of the infrared O-H stretching mode of HOD in a sample of 10 mg DNA in 1 cm$^3$ of 0.4 mol dm$^{-3}$ LiCl(aq) at 218 K

- (A) Sum of bands C and D (infrared O-H stretching band of 10 mg DNA + Gaussian band)
- (B) Infrared spectrum of 0.4 mol dm$^{-3}$ LiCl(aq) + 10 mg DNA
- (C) Infrared spectrum of 10 mg DNA(aq) at 218 K (offset by -10)
- (D) Gaussian band (Offset by -10)
Figure A4  Simulation of the infrared O-H stretching mode of HOD in a sample of 10 mg DNA in 1 ml of 0.6 M LiCl (aq)
Figure A5  Simulation of the infrared O-H stretching mode of isotopically dilute HOD in a sample of 1 cm3 of 0.8 mol dm-3 LiCl(aq) which contains 10 mg DNA

- (A) Sum of bands C and D (infrared O-H stretching band of 10 mg DNA + Gaussian band)
- (B) Infrared spectrum of 0.8 mol dm-3 LiCl(aq) + 10 mg DNA
- (C) Infrared spectrum of 10 mg DNA(aq) at 218 K (offset by -10)
- (D) Gaussian band (offset -10)
Figure A6. Simulation of the O-H infrared absorption of isotopically dilute HOD in D2O in a sample prepared by adding 10 mg DNA to 1 cm$^3$ of 1 mol dm$^{-3}$ LiCl(aq) and then cooling to 218 K.
Appendix B

Infrared Spectra of DNA(aq) + NaCl(aq)

Simulated and "real" infrared spectra of frozen aqueous solutions containing DNA and NaCl (figures B1 - B8).
Figure B1  Simulation of the O-H infrared absorption of HOD in a frozen aqueous (D2O) solution at 218 K. The sample consists of 10 mg DNA in 1 cm3 of 0.1 mol dm-3 NaCl(aq)
Figure B2  Simulation of the O-H infrared absorption of HOD in a frozen aqueous (D2O) solution at 218 K. The sample consists of 10 mg DNA in 1 cm3 of 0.2 mol dm-3 NaCl(aq).
Figure B3  Simulation of the O-H infrared absorption of O-H in frozen aqueous (D2O) solution at 218 K. The sample consists of 10 mg DNA in 1 cm3 of 0.3 mol dm-3 NaCl(aq).

- (A) Sum of bands C and E
- (B) Infrared spectrum of 10 mg DNA + 1 cm3 of 0.3 mol dm-3 NaCl(aq), recorded at 218 K.
- (C) Infrared spectrum, recorded at 218 K, of DNA(aq) (10 mg DNA + 1 cm3 of D2O) offset by -10
- (D) Gaussian band (offset by -10)
- (E) Lorentzian band (offset -10)
Figure B4 Simulation of the infrared absorption of HOD in frozen aqueous (D2O) solution at 218 K. The sample consists of 10 mg DNA in 1 ml of 0.4 mol dm⁻³ NaCl(aq)
Figure B5 Simulation of the O-H stretching mode of HOD in isotopically dilute (D2O) solution at 218 K. The sample consists of 10 mg DNA in 1 cm³ of 0.5 mol dm⁻³ NaCl(aq)
Figure B6 Simulation of the O-H stretching mode of HOD in isotopically dilute (D2O) solution at 218 K. The sample consists of 10 mg DNA added to 1cm3 of 0.6 mol dm-3 NaCl(aq)

- (A) Sum of bands C and E
- (B) Infrared spectrum of 10 mg DNA + 1 cm3 of 0.6 mol dm-3 NaCl(aq), recorded at 218 K
- (C) Infrared spectrum recorded at 218 K, of DNA(aq) (10 mg DNA + 1 cm-3 of D2O) offset by -10
- (D) Gaussian band (offset -10)
- (E) Lorentzian band (offset -10)
Figure B7  Simulation of the infrared O-H stretching mode of HOD in isotopically dilute D2O for a sample of 10 mg DNA in 1 ml of 0.7 M NaCl

- (A) Sum of bands C and E
- (B) Infrared spectrum of 10 mg DNA + 1 cm$^3$ of 0.7 mol dm$^{-3}$ NaCl(aq) at 218 K
- (C) Infrared spectrum, recorded at 218 K, of DNA(aq) (10 mg DNA + 1 cm$^3$ of D2O) offset by -10
- (D) Gaussian band (offset -10)
- (E) Lorentzian band (offset -10)
Figure B8 Simulation of the infrared O-H stretching mode of HOD isotopically dilute in D2O for a sample of 10mg DNA in 1 cm3 of 0.8 mol dm-3 NaCl(aq), recorded at 218 K.
3.7 References


Chapter 4

Sodium-23 NMR Studies Of Gamma Irradiated DNA
Sodium-23 NMR Studies Of Gamma Irradiated DNA

This chapter reports Na-23 nuclear magnetic resonance (NMR) spectra of gamma irradiated solutions containing the sodium salt of DNA (NaDNA(aq)). With such solutions, a decrease in Na-23 linewidth with increasing radiation dose is generally observed. The reduction in the width at half height (v) of the Na-23 resonance has been accounted for in terms of radiation induced strand breaks on DNA. This reduction in linewidth therefore provides a non-intrusive method for measuring strand breaks.

Strand breaks in native (double stranded) DNA and also in thermally denatured (single stranded) DNA relieve intramolecular phosphate–phosphate coulombic repulsions by cleaving the DNA sugar phosphate chain perpendicular to the z axis. This DNA fragmentation is accompanied by the release of sodium counter-ions from the vicinity of DNA into bulk solution. Previous (conductimetric) studies have shown that, with single stranded DNA, 15 sodium ions are liberated from the vicinity of each break in the DNA strand [1].

The work presented in this chapter confirms that, on average, approximately 15 sodium ions are liberated from the vicinity of every strand break in single stranded DNA and indicates that Na-23 NMR spectroscopy may be used to calculate the number of DNA strand breaks in aqueous solutions containing single stranded DNA. In addition, Na-23 NMR line narrowing has been used to determine the number of sodium counter-ions released from native (double stranded) DNA per single strand break.

Sodium NMR experiments using irradiated solutions of double stranded DNA indicate that Na-23 NMR may be used to calculate the number of single strand breaks in double stranded DNA in aqueous solution.
DNA Strand Breaks

Strand breaks result from cleavage of the sugar-phosphate backbone within the DNA molecule. A single strand break (ssb) in DNA results from a single lesion to one of the sugar-phosphate chains. A double strand break (dsb) occurs when the two opposite polynucleotide strands in DNA are cleaved and the molecule is split perpendicular to the z axis.

The majority of radiation induced DNA damage produced in dilute (fluid) aqueous solution arises from radiolysis products of water. When DNA is irradiated in aqueous solution the yield of single strand breaks increases linearly with dose. In contrast, the yield of double strand breaks shows a quadratic dependence on dose (the square root of the double strand break yield increases linearly with dose) [2]. From these dose-yield relationships, early studies concluded that double strand breaks are formed from the coincidence of two single strand breaks on opposite strands of the double helix [2,3]. This 'two hit' model for double strand break formation assumes that when a sufficiently large number of single strand breaks accumulate in the two polynucleotide strands of a DNA molecule, it is highly probable that a single strand break on one polynucleotide strand is close to a single strand break on the opposite strand. Hence accumulation of single strand breaks results in double strand breaks on DNA.

More recent studies of strand breaks in DNA irradiated in (fluid) aqueous solution have found a linear dose-yield contribution to the quadratic relationship of double strand break formation with increasing radiation dose. This linear component is attributed to a 'single hit' process. In aqueous solution, single strand breaks and double strand breaks are generated mainly by OH° radical attack on DNA. Siddiqi and Bothe [4] studied single strand break and double strand break formation in irradiated DNA(aq) solutions, describing the dependence of strand breaks on radiation dose and OH° radical scavenger concentration. Solutions were saturated with N₂O(g) or...
\[ \text{N}_2\text{O}(g) + \text{O}_2(g) \rightarrow \text{N}_2\text{O}(g)/\text{O}_2(g) = 80/20). \] Under these conditions solvated electrons, formed by the radiolysis of water, are converted into \( \text{OH}^\circ \) by reaction with \( \text{N}_2\text{O} \) and water:

\[ \text{e}^- (\text{aq}) + \text{N}_2\text{O}(\text{aq}) + \text{H}_2\text{O} \rightarrow \text{N}_2(g) + \text{OH}^\circ (\text{aq}) + \text{OH}^- (\text{aq}) \]

The extent of double strand breaks was not linearly dependent on dose and the data were analysed in terms of "double strand break concentration", using the following equation:

\[ [\text{dsb}] = \alpha D - \beta D^2 \]

\( D \) is the absorbed dose, \( \alpha \) is the linear component and \( \beta \) is the quadratic component in the dose-effect relationship.

The quadratic component (\( \beta \)) was interpreted in terms of the two hit model where double strand breaks are produced through the accumulation of single strand breaks. For oxygenated \( \text{N}_2\text{O} \) saturated solutions containing DNA, \( \beta \) was \( 2.15 \times 10^{14} \) mol dm\(^{-3}\) Gy\(^{-2}\) and \( \alpha \) was \( 4.1 \times 10^9 \) mol dm\(^{-3}\) Gy\(^{-1}\). In order to ensure that \( \alpha \) measured only radiation induced double strand breaks, the number of single strand breaks present prior to the irradiation of DNA samples was determined. Single strand breaks that were not radiation-induced were found to account for approximately 20% of the observed \( \alpha \) values. The corrected \( \alpha \) value was \( 3.2 \times 10^9 \) mol dm\(^{-3}\) Gy\(^{-1}\). Thus a proportion of double strand breaks were due to single radiation induced events via a 'one hit' mechanism.

Irradiation of either dry DNA or DNA in frozen aqueous solution also yields single strand breaks and double strand breaks. In these systems, where direct radiation damage mechanisms dominate, the yields of both single strand breaks and double strand breaks increase linearly with dose [5].

### 4.1.2 Sodium-23 NMR Spectroscopy

Na-23 NMR relaxation rates (transverse or longitudinal) are considerably enhanced in solutions which contain polyanions such as
DNA. This enhancement is reflected in Na-23 NMR linewidths, which are approximately 7 Hz for 0.02 mol dm$^{-3}$ NaCl(aq) solutions and approximately 49 Hz for NaDNA(aq) solutions which have a similar sodium ion concentration i.e. for solutions prepared by adding 1 cm$^{-3}$ of water to 5 mg highly polymerised sodium salt of calf thymus DNA.

Na-23 nuclei are quadrupolar (nuclear spin I = 3/2) and the interaction of Na-23 quadrupole moments with fluctuating local electric field gradients dominate Na-23 NMR relaxation processes in solutions which contain NaDNA(aq). The observed relaxation rate is a weighted average of the local relaxation rates of all distinct molecular environments among which Na-23 nuclei rapidly exchange.

The Two State Na$^{+}$(aq) Model

Na-23 NMR studies of cation-DNA interactions generally employ a two state model in which Na$^{+}$(aq) ions are considered to exchange rapidly between two distinct environments which are termed 'bound' and 'free' [6,7]. In the 'bound' state, Na$^{+}$(aq) ions are held close to the DNA surface, and territorially bound to DNA by electrostatic attraction. Bound Na$^{+}$(aq) interacts with the electric field gradient of DNA but is not localised at specific sites on DNA. The 'free' state is equivalent to Na$^{+}$(aq) in the bulk aqueous phase, where quadrupolar interactions between sodium ions and DNA do not occur. Thus the observed relaxation rate, $R_{\text{obs}}$, is related to the relaxation rates of the Na$^{+}$(aq) bound and Na$^{+}$(aq) free ions in solution by the following equation:

$$R_{\text{obs}} = x_{b} R_{b} + x_{f} R_{f}$$

The subscripts b and f refer to bound and free sodium ions respectively, $R$ are the relaxation rates (longitudinal or transverse); $x_{b}$ and $x_{f}$ are mole fractions of bound and free sodium ions.
Quadrupolar Relaxation

Whenever the distribution of electric charge about sodium is asymmetric the quadrupole moment of the sodium nucleus interacts with the electric field gradient. The quadrupole coupling constant \( \chi \) is the product of this interaction and is given by:

\[
\chi = \frac{2\pi e^2 Q q}{h}
\]

Here \( e \) is the elementary charge, \( Q \) is the electric quadrupole moment of the nucleus, \( q \) is the electric field gradient and \( h \) is the Planck constant [8].

In a magnetic field \( B_0 \) a sodium nucleus can assume \((2l+1) = 4\) orientations. The transitions between the corresponding energy states are degenerate in the absence of an electric field gradient \( q \) at the nucleus. If an electric field is present the degeneracy is lifted and three NMR transitions should be detected as three lines, the distance between two adjacent lines being equal and given by:

\[
\text{distance} = 0.25 \chi (3 \cos^2 \theta - 1)
\]

The angle \( \theta \) is that between \( B_0 \) and the direction of the field gradient. However in liquid aqueous solutions, rapid reorientational motions ensure that \( \theta \) fluctuates over all possible values. Thus the three transitions collapse to a single line having a linewidth, \( \nu \), which is related to the quadrupole coupling constant by:

\[
\nu = 0.4 \pi \chi \tau_c
\]

Here \( \tau_c \) is a correlation time which describes the reorientation of the quadrupole interaction between the Na-23 nucleus and the local electric field gradient [8].

Na-23 NMR Linewidths

In general Na-23 line shapes are Lorentzian and are characterised by their linewidth. In the limit of extreme narrowing (where the
longitudinal $T_1$ relaxation time is equal to the transverse $T_2$ relaxation time) the linewidth is related to $T_2$ by:

$$\nu = (\pi T_2)^{-1}$$

However, in Na-23 NMR spectroscopy of NaDNA(aq) solutions at and above field strengths corresponding to a Larmor frequency of 200 MHz for protons (a Larmor frequency for sodium, $\omega_{Na}$, of 52.9 MHz) $T_1$ and $T_2$ relaxation times are not equal and Na$^+(aq)$ NMR spectra are non-Lorentzian [9]. The NMR line shape consists of two components which can be fitted to a combination of two Lorentzian lines; 60% of the Na-23 NMR signal intensity is characterised by a large linewidth and 40% of the total signal intensity is characterised by a relatively small linewidth [10,11]. At high magnetic field strengths, the observed Na-23 NMR linewidth has a more complex relationship to sodium nuclei relaxation times. However, linewidth may be used in a qualitative comparison of sodium nuclei relaxation enhancement. For example Nordenskiöld et al obtained Lorentzian Na-23 NMR spectra of solutions which contained poly d(AT)$_n$ and non-Lorentzian spectra for solutions which contained either poly Br-d(GC)$_n$, or poly d(GC)$_n$. Sodium-23 NMR linewidths were used as a qualitative indication of the relaxation enhancement in the Na$^+(aq)$ 'bound' state [12].

4.1.3 Counter-ion Condensation Theory

In aqueous solutions containing DNA$^-(aq)$ and Na$^+(aq)$, the local counter-ion concentration is high in the immediate vicinity of DNA. In NaDNA(aq)solutions, Na$^+(aq)$ ions cluster close to the DNA(aq) surface as a result of the strong coulombic attraction between Na$^+(aq)$ ions and charges centred on the DNA phosphate groups. This clustering of small counter-ions about large polyions is counter-ion condensation. The molecular theory of counter-ion condensation with applications to polynucleotide solutions is described by Manning [13].

Manning described counter-ion condensation according to the molecular theory of polyelectrolyte solutions, in which DNA in an aqueous solution containing small cations like Na$^+(aq)$ possesses a
saturated charge fraction which is less than one but greater than zero [13]. For polyions in general, this charge fraction is independent of bulk concentration and is dependent on the valence of the counterion, \( N \), and the axial charge density of the polyion. A quantity \( b \) is the average axial charge spacing. A dimensionless parameter \( \xi \) is defined as proportional to the charge density:

\[
\xi = \frac{q^2}{\varepsilon k T b}
\]

Here \( q \) is the charge on a proton, \( \varepsilon \) is the relative permittivity of the solvent, \( k \) is the Boltzmann constant and \( T \) is temperature. Hence the saturated charge fraction is observed to be close to \( (N\xi)^{-1} \).

Manning formulated an operational definition of counter-ion condensation thus: "the mode of binding of counter-ions of valence \( N \) to a polyelectrolyte is called condensation if, in an environment containing only the single counter-ion species, the charge fraction of the polyelectrolyte equals the constant value \( (N\xi)^{-1} \) over a broad concentration range."

For aqueous solutions at 298 K, \( \xi = (7.1/b) \) where \( b \) is expressed in Å. The step height distance between base pairs, and thus between phosphate pairs, in B-DNA is 3.4 Å. Therefore the average axial charge spacing \( b \) is 1.7 Å and \( \xi_{\text{DNA}} = 4.2 \). Thus the saturated charge fraction, \( (N\xi_{\text{DNA}})^{-1} \), of a solution containing NaDNA(aq) is 0.24, i.e. 76% of the phosphate charge in DNA is neutralised by counter-ion condensation. Similarly, for single stranded DNA in aqueous solution at 298 K, \( b \) is 4.0 Å. Hence \( \xi_{\text{ssDNA}} = 1.8 \) and thus \( (N\xi_{\text{ssDNA}})^{-1} = 0.56 \), i.e. 44% of the phosphate charge on single stranded DNA is neutralised by counter-ion condensation.

A high local concentration of Na\(^+\)(aq) is maintained by the DNA system in order to relieve intramolecular phosphate – phosphate electrostatic repulsion. Spontaneous diffusion of 'bound' Na\(^+\)(aq) into bulk solution is opposed by the requirement to minimise the Gibbs energy of interaction between phosphate groups. Hence, any alternative mechanism which results in reducing the intramolecular phosphate – phosphate repulsion would be accompanied by the release of 'bound' sodium ions from the vicinity of DNA into bulk solution. Examples of such alternative mechanisms are:
i. 'Binding' of cations, such as spermine, to DNA. The extent to which cations 'bind' to DNA may be determined by the titration of NaDNA(aq) with polyammonium cations. The polyammonium cations replace 'bound' sodium ions. The release of DNA bound sodium ions into bulk solution with the addition of polyammonium cations may be followed by monitoring the decrease in Na-23 NMR linewidths [14,15,16].

ii. DNA fragmentation; the release of DNA bound sodium ions following radiation induced DNA strand breaks has been monitored conductimetrically [1].

Experimentally, NMR measurements confirm that the number of counter-ions close to a highly charged polyion (i.e. DNA) is independent of the amount of salt in solution over a wide range of salt concentrations. Anderson and co-workers monitored the extent to which sodium ions associate with double stranded DNA by measuring Na-23 linewidths [7]. The concentration dependence of the sodium linewidth was determined by adding NaCl to solutions of tetraethyl- or tetrabutylammonium DNA. At ratios of Na\(^+\)(aq) : DNA phosphate of greater than three ([Na]/[P]>3), only sodium was associated with DNA and the compensated charge on DNA did not vary, even at the highest salt concentration investigated which was equivalent to [Na]/[P] = 30.

At [Na]/[P]<3, the tetraalkylammonium cation competes with Na\(^+\)(aq) for association with DNA. An equation was derived for the effect of this competition on the Na-23 linewidth. This equation fitted all linewidth data if the charge fraction, \(N^\text{DNA}_{\text{Na}}\)^{-1}, was in the range 0.25 ± 0.10. The assumption was made that the fraction of uncompensated charge on DNA remained constant after the 'condensation' of both cations. Both the experimentally derived value of \(N^\text{DNA}_{\text{Na}}\)^{-1}, and its lack of sensitivity with respect to large variations in the concentration of added salt, are in good agreement with counter-ion condensation theory.
4.2 Previous Work

Double and single strand breaks in irradiated DNA(aq)

Hagen [2] determined the yield of single and double strand breaks in irradiated DNA by studying the change in distribution of the DNA molecular mass following irradiation. Solutions containing 0.2 mg cm⁻³ double stranded DNA were irradiated in aqueous solution. These DNA solutions were not conditioned by treatment with gases and contained dissolved air.

Samples containing double stranded DNA were placed in an ultracentrifuge and DNA sedimentation coefficients, S, were measured. Histograms of the fraction of molecules with a particular S were produced. For a particular sample, an average S was determined from the corresponding histogram. The average molecular mass of double stranded DNA, \( M_{dsDNA} \), was calculated from the average sedimentation coefficient, S, using the empirically derived relationship:

\[
S = 0.063 M_{dsDNA}^{0.37}
\]

The yield of double strand breaks per DNA base pair was calculated from the average yield of DNA fragments. These double strand break yield results are shown in figure 4.1.

Single strand break yields in DNA were calculated by denaturing the double stranded DNA following irradiation. A DNA solution was mixed with 0.2 mol dm⁻³ NaOH(aq) and then a 37% formaldehyde solution added. After 30 seconds the resulting solution was neutralised with KH₂PO₄(aq) and brought to the required concentration by addition of 0.2 mol dm⁻³ NaCl(aq).

An ultracentrifuge was also used to determine the sedimentation coefficients, S, for the single stranded DNA samples. Histograms of the fraction of molecules with particular values of S were produced and the molecular mass, \( M_{ssDNA} \), of single stranded DNA within a
Figure 4.1a Dose-yield plot for the formation of single strand breaks from double stranded DNA irradiated in aqueous solution. Adapted from Hagen [2].

Figure 4.1b Dose-yield plot for the formation of double strand breaks from double stranded DNA irradiated in aqueous solution. Adapted from Hagen [2].
particular sample was determined using the following empirical relationship:

\[ S = 0.057 M_{ssDNA}^{0.37} \]

Hagen calculated the number of single strand breaks per nucleotide from the ratio of the yield of single stranded DNA molecules after irradiation to the number of single stranded DNA molecules in unirradiated samples containing single stranded DNA. These results are also shown in figure 4.1.

The release of DNA counter-ions following irradiation

Na-23 NMR spectroscopy has been used to investigate the origin of conductivity changes to DNA solutions following strand break formation [1]. In these experiments, by Bothe et al, NMR spectra were recorded using a Bruker WH270 FT NMR spectrometer. For unirradiated samples, which contained 260 mg dm$^{-3}$ double stranded DNA (dsDNA(aq)), the Na-23 NMR linewidths ($\nu$) were $46 \pm 4$ Hz. With irradiated samples containing double stranded DNA(aq), $\nu$ decreased with increasing radiation dose.

The two state model in which Na$^+(aq)$ ions are described as either 'bound' or 'free' was used to relate the fraction of free Na ions ($\alpha$) to the observed linewidth. Thus:

\[ \alpha = \frac{\nu_{\text{obs}} - \nu_b}{\nu_f - \nu_b} \]

Here $\nu_{\text{obs}}$ is the observed Na-23 NMR linewidth, $\nu_f$ is the linewidth of free Na$^+(aq)$ and $\nu_b$ is the linewidth of bound Na$^+(aq)$. The value of $\nu_f$ was taken as $12.3 \pm 1$ Hz which was the Na-23 linewidth obtained by NMR of a solution containing a mixture of the sodium salts of 2'-deoxyadenosine 5'-monophosphate and 2'-deoxyguanosine 5'-monophosphate. The linewidth of 'bound' sodium ($\nu_b$) was calculated using NMR spectra in conjunction with conductivity measurements.

Bothe et al took conductivity measurements of samples which contained either single stranded DNA (ssDNA) or double stranded
DNA (dsDNA). Samples were prepared as N$_2$O saturated, aqueous solutions. Both ssDNA and dsDNA solutions were irradiated at 293 K with Co-60 gamma radiation. The conductivity of DNA solutions was measured before irradiation ($\chi_0$) and after irradiation ($\chi$).

For solutions which contain ssDNA, the conductivity increased ($\chi > \chi_0$) with increasing radiation dose. Solutions containing 28 mg dm$^{-3}$ DNA released approximately 15 'DNA bound' sodium ions per single strand break. A previous study [17] had determined $G$ for single strand break formation in single stranded DNA irradiated in N$_2$O saturated aqueous solution, $G_{ssb} = 0.55$. $G$ for Na release ($G_{Na^+}$) from single stranded DNA was therefore calculated as $15 \times G_{ssb} = 8.3$.

For solutions containing dsDNA which were irradiated to 30 Gy the conductivity post irradiation decreased by approximately 0.5%. The conductivity of dsDNA(aq) samples irradiated to doses greater than 30 Gy increased dramatically. At doses greater than 400 Gy the increase in conductivity was less pronounced and a plateau was approached at approximately 1.5 kGy. Conductivity measurements for dsDNA(aq) solutions, normalised to the conductivity of a dsDNA solution irradiated to 2 kGy ($\chi_{max}$), were used to define the dependence of $\alpha$, the fraction of free Na$^+(aq)$, on conductivity:

$$\alpha = \frac{\chi}{\chi_{max}}$$

The 'bound' Na-23 NMR linewidth $\nu_b$ was calculated by substituting the value of $\alpha$ at $\chi = \chi_0$ into the derived equation for the dependence of $\alpha$ with NMR linewidth. A plot of $\alpha$ derived from the normalised increase in conductivity ($\alpha = \chi/\chi_{max}$) as a function of radiation dose and a plot of $\alpha$ derived from Na-23 NMR linewidth measurements as a function of dose gave very similar curves (figure 4.2). This result indicated that the conductivity increase was caused by the transition of Na$^+(aq)$ ions from the DNA 'bound' state to the 'free' state.
Figure 4.2 Relative increase of conductivity $\kappa (\infty)$ and relative decrease of Na-23 NMR signal line width ($xx$) as a function of dose measured after Co-60 gamma irradiation in $N_2O$ saturated aqueous solutions of DNA (260 mg l$^{-1}$). $\kappa$ and $\Delta \nu$ are the conductivity and the linewidth observed with solutions at the respective doses. $\kappa_{\text{max}}$ is the plateau conductivity at doses $\geq 1.5$ kGy, $\Delta \nu_B$ and $\Delta \nu_f$ denote the relative linewdths of the DNA "bound" and "free" Na ions [1].
4.3 Experimental

4.3.1 Materials

The highly polymerised sodium salt of Calf Thymus Deoxyribonucleic acid (DNA) was purchased from BDH Chemicals Limited. Unless stated otherwise, DNA of a single batch number was used throughout each experiment. All reagents used were of the highest grade available. Deuterium oxide (D₂O 99.9% D) was used as purchased from Goss Limited.

4.3.2 Preparation Of Samples

As purchased, DNA contains excess sodium chloride. Prior to sample preparation this excess was removed using dialysis. Typically 25 cm³ of water was added to 0.5 g of DNA and left for approximately 24 hours at approximately 4 °C to ensure that the DNA was fully hydrated. The resulting solution was transferred by syringe into cellulose tubing which was sealed and dialysed against water for at least 12 hours at approximately 4 °C. The sodium salt of DNA was recovered from solution by freeze drying and was then stored in sealed sample tubes at approximately 4 °C until required.

DNA samples were prepared as bulk stock solutions. A known volume of water was added to a known weight of DNA. Solutions containing single stranded DNA were prepared by thermally denaturing a solution of Calf Thymus DNA. A stock DNA solution was heated to approximately 90 °C for five minutes and then rapidly cooled using an ice bath [1].

Aliquots of approximately 1 cm³ of stock DNA solution were placed into sample tubes which were sealed and irradiated. After irradiation the samples were stored at approximately 4 °C until required for NMR measurements. DNA samples were transferred into NMR tubes by syringe. Air bubbles, if formed, were removed by gentle centrifugation.
In experiments which compared Na-23 NMR linewidths derived from single stranded and double stranded DNA solutions, samples were prepared by adding 125 mg double stranded DNA (dsDNA) to 25 cm$^3$ of water (10 cm$^3$ D$_2$O + 15 cm$^3$ H$_2$O). The stock solution was divided into two parts. One part was thermally denatured to produce single stranded DNA (ssDNA). The remaining DNA solution was not thermally denatured and contained DNA in the native, double stranded, state. Two sets of samples were produced from these stock solutions and were co-irradiated by exposure to Co-60 gamma radiation (dose rate = 0.64 Gy s$^{-1}$). One set of five samples were each equivalent to 5 mg ssDNA plus 1 cm$^3$ water, and the other set of six samples were equivalent to 5 mg dsDNA plus 1 cm$^3$ water.

4.3.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) spectra were obtained using a Bruker AM300 Fourier Transform spectrometer. This NMR spectrometer operated at a Larmor frequency of 79.39 MHz for Na-23. The probe heads were equipped for an internal deuterium lock. Typically Na-23 NMR spectra of solutions containing DNA were recorded at 298 K after accumulation of 5000 pulses of 14 $\mu$s duration with an acquisition time of 0.3 s. The sweep width was 1259.5 Hz and spectra were zero-filled to 16 k data points prior to Fourier Transformation. An Aspect 300 microcomputer was used to drive the spectrometer and to process the recorded spectra.

4.3.4 Gamma Radiation Source

Samples were gamma irradiated using a Co-60 source. The radiation source comprised of pellets of radioactive Co-60 metal arranged cylindrically about an inner cavity. This assembly was buried in a deep concrete-lined pit. The entrance to the irradiation chamber was shielded from the operator by a large lead cabinet. Samples were placed into the irradiation chamber using a piston driven by compressed air.
4.4 Results & Discussion

4.4.1 Irradiation Of Single Stranded And Double Stranded DNA

A plot of the Na-23 NMR linewidth at half height against dose for both ssDNA and dsDNA samples is given in figure 4.3.

Samples containing single stranded DNA show a reduction in Na-23 NMR linewidth at half height (VssDNA) with increasing dose in the dose range between 0 and approximately 600 Gy (figure 4.3). Linear regression of the data points for single stranded DNA produces a gradient of VssDNA with dose as $-1.2 \times 10^{-3}$ Hz Gy$^{-1}$ with an intercept of 20.8 Hz.

The change in width at half height of the Na-23 resonance of samples containing double stranded DNA (VdsDNA) is less than that observed for single stranded DNA in the same dose range. Na-23 linewidth data for dsDNA, as a function of dose, were fitted to a straight line; linear regression yields a gradient of $-6 \times 10^{-3}$ Hz Gy$^{-1}$ and an intercept of 48.9 Hz (figure 4.3).

Sodium chloride and sodium deoxyguanosine monophosphate

Na-23 NMR linewidths were measured for solutions containing increasing amounts of NaCl(aq). The Na-23 NMR linewidth of approximately 7.3 Hz was independent of NaCl(aq) concentration in the range 0.02 mol dm$^{-3} < [\text{NaCl}] < 0.1$ mol dm$^{-3}$ (figure 4.4). A separate set of Na-23 NMR spectra were recorded for solutions containing increasing amounts of a DNA nucleotide, the sodium salt of 2'-deoxyguanosine 5'-monophosphate (dGMP). The resulting NMR linewidths are also presented in figure 4.4.

4.4.2 Discussion

In the two state model in which sodium ions rapidly exchange between DNA bound and DNA free states the observed NMR linewidth is related to the number of bound and free sodium ions by:
Figure 4.3  Na-23 NMR linewidths of irradiated solutions containing (i) double stranded DNA and (ii) single stranded DNA. Each DNA sample was equivalent to 5 mg NaDNA added to 1 cm3 of water.
Figure 4.4 Na-23 NMR Spectral linewidths of solutions containing (i) NaCl(aq) and (ii) the sodium salt of deoxyguanosine 5'-monophosphate, Na(dGMP), plotted as a function of the number of sodium ions contained in 1 cm$^3$ of solution.
\begin{equation}
N V_{\text{obs}} = n_b V_b + n_f V_f \tag{1}
\end{equation}

Here $V$ are Na-23 linewidths; $V_{\text{obs}}$ refers to the observed Na-23 NMR linewidth, and $V_b$ and $V_f$ refer to the Na-23 NMR linewidth of sodium ions in the DNA bound and free states respectively. $N$ is the total number of sodium ions in solution, $n_b$ and $n_f$ are the number of bound and free sodium ions respectively ($n_b + n_f = N$).

From equation (i):

\begin{equation}
\frac{n_b}{N} = \frac{(V_{\text{obs}} - V_f)}{(V_b - V_f)} \tag{ii}
\end{equation}

\begin{equation}
x_b = \frac{(V_{\text{obs}} - V_f)}{(V_b - V_f)} \tag{iii}
\end{equation}

Thus $n_b/N$ is the mole fraction of bound sodium ions, $x_b$. The linewidth of free sodium ions, $V_f$, was estimated by inspection of figure 4.4 as 7.3 Hz; i.e. equal to the linewidth of sodium ions in an aqueous solution of sodium chloride.

4.4.3 Single Stranded DNA

According to counter-ion condensation theory the number of sodium ions bound to single stranded DNA in aqueous solution is 0.44 sodium ions per DNA phosphate [13]. Thus $x_b = 0.44$ for unirradiated ssDNA. Therefore $x_f = 0.56$ because in these DNA samples the total number of sodium ions is equivalent to the total number of DNA phosphate groups.

The linewidth, $V_b$, for unirradiated solutions containing ssDNA has been calculated as follows:

\begin{equation}
V_b \text{ (ssDNA)} = \frac{(V_{\text{obs}} - x_f V_f)}{x_b} = \frac{[20.8 \text{ Hz} - 0.56 (7.3 \text{ Hz})]}{0.44} = 38.0 \text{ Hz}
\end{equation}

The linewidth, $V_{\text{obs}}$, was taken to be the intercept in figure 4.3 for samples containing single stranded DNA. The mole fractions $x_f$ and
$x_b$ are those calculated by counter-ion condensation theory [13] and the linewidth of free sodium ions, $V_f$, was taken to be equal to the linewidth of sodium ions in an aqueous solution of sodium chloride (figure 4.4).

Assuming that $V_b$ and $V_f$ remain constant over the dose range studied, the number of sodium ions released from the DNA bound state to the free state per unit dose may be calculated from the gradient of a plot of the change in Na-23 NMR linewidth with dose. The following equation has been derived in appendix A and describes the change in Na-23 NMR linewidth with increasing radiation dose in terms of the net release of sodium ions from the bound DNA state to the free DNA state, thus:

$$V_{\text{obs}}^0 - V_{\text{obs}}' = \lambda D (V_b - V_f)$$

Here $V_{\text{obs}}^0$ is the observed Na-23 NMR linewidth of unirradiated DNA samples, $V_{\text{obs}}'$ is the observed linewidth of irradiated DNA samples, $D$ is absorbed dose and $\lambda$ is the number of sodium ions per DNA phosphate released from the bound to the free state (Ar) per unit of absorbed dose. Hence a plot of $V_{\text{obs}}^0 - V_{\text{obs}}'$ against dose, $D$, is a straight line, gradient equal to $\lambda (V_b - V_f)$.

Although, following irradiation, the bulk solution will contain fragments of DNA, the assumption that $V_f$ remains constant for DNA samples irradiated to doses less than 600 Gy is reasonable. The single stranded DNA samples each contained approximately $1.51 \times 10^{-5}$ moles of DNA phosphate. In figure 4.4 a Na-23 NMR linewidth of 7.9 Hz for a solution containing $1.51 \times 10^{-5}$ moles of Na(dGMP) may be obtained by extrapolation. This linewidth is 0.6 Hz greater than that of NaCl(aq). Therefore the assumption that $V_f$ remains constant up to a dose of 600 Gy is valid. Even if all DNA nucleotide units were released into bulk solution the linewidth of free Na$^+$ (aq) would change by less than 1 Hz.

The linewidth $V_b$ should remain constant at low radiation doses where DNA counter-ion condensation operates. However, following irradiation to sufficiently high doses, the DNA in solution will become
severely fragmented. Kleman and Leyte [18] showed that for polyphosphate solutions counter-ion condensation does not occur if the polyphosphate chain length falls below a critical value which corresponds to a degree of polymerisation of approximately 60. Therefore counter-ion condensation should cease to operate when the molecular weight of DNA falls below a critical value. Sodium NMR experiments, involving solutions with a concentration of DNA equivalent to 10 mg DNA added to 1 cm$^3$ of water, show that the Na-23 NMR linewidth narrowing proceeds in a linear fashion with dose in the dose range 0 - 1 kGy. Above 1 kGy the Na-23 NMR linewidth becomes narrower at a more reduced rate with increasing radiation dose and begins to plateau at approximately 2.5 kGy (figure 4.5). Presumably, above a radiation dose of 1 kGy, the average DNA chain length is less than that required to maintain counter-ion condensation.

### The number of sodium ions released per Gray of absorbed dose

Values of $V^o_{obs} - V'_{obs}$ have been calculated for solutions containing single stranded DNA. The linewidth $V^o_{obs}$ corresponds to that of unirradiated single stranded DNA and was taken as the intercept of the plot of the Na-23 NMR linewidth of single stranded DNA against dose in figure 4.3. The change in Na-23 NMR linewidth on irradiation ($V^o_{obs} - V'_{obs}$) is plotted against absorbed dose in figure 4.6. Linear regression of the data points in figure 4.6 used an intercept fixed at 0 Hz. The calculated gradient of the line is $1.24 (\pm 0.15) \times 10^{-2}$ Hz Gy$^{-1}$.

Hence, for single stranded DNA:

\[
\lambda(V_b - V_f) = 1.24 (\pm 0.15) \times 10^{-2} \text{ Hz Gy}^{-1}
\]

\[
V_b = 38.0 \text{ Hz}
\]

\[
V_f = 7.3 \text{ Hz}
\]

\[
\therefore \quad \lambda = 0.0124 / 30.7
\]

\[
\lambda = 4.04 (\pm 0.50) \times 10^{-4} \text{ Gy}^{-1}
\]
Figure 4.5 Narrowing of the Na-23 NMR linewidth following irradiation of solutions containing double stranded DNA. The line has been fitted by linear regression of the first 9 data points obtained with solutions containing 10 mg DNA (triangles).

- 5 mg double stranded DNA added to 1 cm³ water
- 10 mg double stranded DNA added to 1 cm³ water

Gradient = 5.10 E-3 (+/- 0.83 E-3)
Figure 4.6 Na-23 NMR linewidth of irradiated DNA samples (V') subtracted from the linewidth of unirradiated DNA samples (Vo) plotted against radiation dose.
Thus with single stranded DNA, \(4.04 \times 10^{-4}\) sodium ions per DNA phosphate are released per Gray of absorbed radiation dose. The error associated with \(\lambda\) has been estimated as \(5 \times 10^{-5}\) Gy\(^{-1}\).

**The number of sodium ions released per strand break**

DNA single strand break data from a study by Hagen [2] was used to calculate the number of radiation induced single strand breaks per DNA nucleotide. Hagen irradiated double stranded DNA and calculated the number of single strand breaks by first denaturing DNA using alkali solution and heat to produce single stranded DNA. A number of studies have shown that, following the irradiation of DNA, there are alkali-labile sites within DNA which produce single strand breaks on treatment with alkali [19,20,21]. In general, for DNA which has been treated with alkali, the single strand break yield is increased by one third. Two thirds of the single strand break yield is a direct result of irradiation and one third of the single strand break yield arises indirectly from radiation damaged sites on DNA via treatment with alkali. For example native (double stranded) DNA irradiated in aerated solutions and treated with 0.1 mol dm\(^{-3}\) NaOH for 3 minutes contains 1 alkali labile site for every two single strand breaks [22].

In order to make a direct comparison between data for single stranded DNA derived from Na-23 NMR linewidth studies (in which DNA was thermally denatured and not denatured by exposure to alkali), and strand break dose - yield data produced by Hagen, the yield of DNA single strand breaks has been reduced by one third. In this way strand breaks generated by the addition of alkali following irradiation are not included. This procedure is necessary because Hagen first irradiated native DNA and then produced single stranded DNA using alkali whereas in the work presented in this chapter, native DNA was thermally denatured to give single stranded DNA and this single stranded DNA was then irradiated.

Figure 4.7 is a plot of the yield of single strand breaks per DNA nucleotide as adapted (as described above) from the data published by Hagen [2]. Linear regression of the data in figure 4.7 yields a
Figure 4.7  Radiation induced single strand breaks in double stranded DNA.
Data adapted from [2] to exclude single strand breaks resulting from treatment of DNA with alkali solution following irradiation.

- Single strand break data adapted from Hagen [2]
- Gradient = 2.35 E-5 (+/- 0.18 E-5)
gradient of $2.35 \pm 0.18 \times 10^{-5}$ Gy$^{-1}$; i.e. the ratio of single strand breaks to DNA nucleotides is $2.35 \pm 0.18 \times 10^{-5}$ Gy$^{-1}$.

The number of DNA nucleotides is equivalent to the number of DNA phosphate groups and thus the yield of DNA single strand breaks per DNA nucleotide is equivalent to the number of DNA single strand breaks per DNA phosphate. Dividing $\lambda$ (the number of sodium ions per DNA phosphate released per Gray of absorbed dose) by the number of single strand breaks per Gray of absorbed dose yields the number of sodium ions released from the DNA bound state per single strand break as a ratio of sodium ions to DNA phosphate in solution.

For single stranded DNA, $\lambda$ equals $4.04 \pm 0.50 \times 10^{-4}$ Gy$^{-1}$. Strand break data adapted from that published by Hagen [2] gives the ratio of single strand breaks to DNA phosphate as $2.35 \pm 0.18 \times 10^{-5}$ Gy$^{-1}$. Thus the ratio of sodium ions per DNA phosphate ($\Delta r$) released per single strand break ($n_{ssb}$) is:

$$\frac{\Delta r}{n_{ssb}} = \frac{4.04 \pm 0.50 \times 10^{-4}}{2.35 \pm 0.18 \times 10^{-5}}$$

$$= 17.2 \pm 2.5 \text{ Na}^+(\text{aq}) \text{ per strand break}$$

For single stranded DNA, Bothe et al determined that 15 sodium ions are released from the DNA bound state to the free state per strand break [1]. The estimate, $17.2 \pm 2.5$ sodium ions released from single stranded DNA per strand break, is in good agreement with that determined by Bothe et al [1] and shows the usefulness of the Na-23 NMR technique for determining the amount of sodium ions released from single stranded DNA and thus the number of strand breaks.

4.4.4 Double Stranded DNA

The release of DNA bound sodium counter-ions following irradiation of double stranded DNA has been monitored by NMR spectroscopy [1]. However, for double stranded DNA, the number of counter-ions released following irradiation was not correlated to strand breaks.
Strand breaks in single stranded DNA can be correlated to the number of sodium ions released from the DNA bound state into bulk solution. With irradiated samples containing double stranded DNA it is reasonable to assume that the number of sodium ions released from the 'DNA bound' to the 'DNA free' state is proportional to the number of DNA double strand breaks and/or the number of DNA single strand breaks induced by irradiation.

In figure 4.3 the dependence on radiation dose of the Na-23 NMR linewidth observed for samples containing double stranded DNA was fitted to a straight line. Hagen's data (figure 4.1) shows that the yield of double strand breaks has a quadratic relationship with radiation dose. However, in the dose range 0 - 400 Gy, the yield of double strand breaks as a function of radiation dose approximates to a straight line. The scatter of the Na-23 NMR data points (figure 4.3) means that interpretation is difficult. These Na-23 NMR results do not show conclusively whether Na-23 NMR linewidth measurements, of solutions containing double stranded DNA are sensitive to double strand breaks, single strand breaks or a combination of both.

The data for double stranded DNA were analysed in a similar way to that used previously for single stranded DNA. The number of sodium ions released following irradiation was calculated in terms of single strand breaks on DNA. In the following section data are presented to support the hypothesis that Na-23 NMR line narrowing is a result of DNA single strand breaks and not radiation induced double strand breaks.

**Calculation of \( v_b \) for unirradiated double stranded DNA(\( \text{aq} \))**

Counter-ion condensation theory requires 0.76 bound sodium ions per DNA phosphate and 0.24 free sodium ions per DNA phosphate [13]. The Na-23 NMR linewidth for free sodium ions was taken as being 7.3 Hz; i.e. the same as for solutions containing single stranded DNA. The observed Na-23 linewidth, \( v_{obs} \), for unirradiated DNA was taken as the intercept in figure 4.3 for samples containing double stranded DNA.
Hence \[ V_b (\text{dsDNA}) = \frac{(V_{obs} - x)N_f}{x_b} \]
\[ = \frac{[48.9 \text{ Hz} - 0.24 (7.3 \text{ Hz})]}{0.76} \]
\[ = 62.0 \text{ Hz} \]

Values of \( V^o_{obs} - V'_{obs} \) were calculated for solutions containing double stranded DNA. The linewidth \( V^o_{obs} \) corresponds to that of unirradiated double stranded DNA and is the intercept of the plot of Na-23 NMR linewidth for double stranded DNA against dose described in figure 4.3.

The narrowing of Na-23 NMR linewidth \( (V^o_{obs} - V'_{obs}) \) on irradiation of double stranded DNA is plotted against absorbed dose in figure 4.6. Linear regression of the data points in figure 4.6 with the intercept fixed at 0 Hz, shows that the gradient of the line is 6.1 \( \pm 1.2 \) x \( 10^{-3} \) Hz Gy\(^{-1}\).

Hence, for double stranded DNA:

\[ \lambda(V_b - V_f) = 6.1 (\pm 1.2) \times 10^{-3} \text{ Hz Gy}^{-1} \]
\[ V_b = 62.0 \text{ Hz} \]
\[ V_f = 7.3 \text{ Hz} \]

\[ \therefore \lambda = 6.1 (\pm 1.2) \times 10^{-3} / 54.7 \]
\[ \lambda = 1.12 (\pm 0.22) \times 10^{-4} \text{ Gy}^{-1} \]

Thus for double stranded DNA, \( 1.12 \times 10^{-4} \) sodium ions per DNA phosphate are released per Gray of absorbed radiation dose. The error associated with \( \lambda \) is estimated as \( 2.2 \times 10^{-5} \) Gy\(^{-1}\).

**The number of sodium ion released per DNA strand break**

Assuming that line narrowing is predominantly due to single strand breaks, the number of sodium ions released per single strand break is calculated by dividing \( 1.12 \times 10^{-4} \) sodium ions per DNA phosphate by the number of single strand breaks per DNA nucleotide i.e. \( 2.35 (\pm 0.18) \times 10^{-5} \text{ Gy}^{-1} \):
\[ \frac{1.12 \pm 0.22 \times 10^{-4}}{2.35 \pm 0.18 \times 10^{-5}} = 4.8 \pm 1.0 \text{Na}^+ \text{(aq)} \text{per DNA phosphate} \]

If radiation induced single strand breaks produces Na-23 NMR line narrowing following irradiation then 4.8 (\pm 1.0) sodium ions per DNA phosphate are released from the DNA bound state to the free state per single strand break in double stranded DNA.

4.4.5 NMR Of More Concentrated DNA Solutions

The experiments which involved irradiation of solutions containing double stranded DNA were repeated with samples equivalent to 10 mg DNA added to 1 cm\(^3\) of water (twice the amount of DNA per sample than used in the experiments reported in section 4.4.1 and discussed in section 4.4.4). Samples were irradiated over a larger dose range than those reported earlier on in section 4.4.1. In figure 4.6 the two sets of NMR linewidth data are summarised. One set is that reported earlier and has a DNA concentration equivalent to 5 mg DNA added to 1 cm\(^3\) of water. The other set of data in figure 4.6 is derived from samples having a DNA concentration equivalent to 10 mg DNA added to 1 cm\(^3\) of water.

Linear regression of the first nine data points for samples with the higher DNA concentration was performed with the intercept fixed at 0 Hz. The gradient of the line is 5.10 (\pm 0.83) \times 10^{-3} \text{Hz Gy}^{-1}. Hence 3.97 (\pm 0.71) sodium ions are released from the DNA bound state, per single strand break in double stranded DNA.

4.4.6 Double Strand Breaks And Sodium Ion Release

Radiation induced DNA double strand break data have been interpreted as having both linear and quadratic components [4]. The yield of double strand breaks per DNA nucleotide, \(\text{DSB}^\circ\), is related to absorbed dose D according to:

\[ \text{DSB}^\circ = \alpha D + \beta D^2 \]
Here $\alpha$ is the linear component and $\beta$ is the quadratic component of the dose-yield relationship. A plot of $\text{DSB}^o/D$ versus $D$ produces a straight line of gradient $\beta$ and intercept $\alpha$ (figure 4.8).

Similarly, the Na-23 NMR linewidth narrowing, observed with irradiated DNA(aq) solutions, should have the same dose–effect relationship if the NMR line narrowing originated solely from radiation induced double strand breaks. In other words the Na-23 NMR linewidth of irradiated DNA samples subtracted from that of an unirradiated sample, $V^{\text{obs}}_o - V^{\text{obs}}_i$, divided by dose plotted against the same dose should yield a gradient $b$ and an intercept $a$. Here $a$ is the linear component and $b$ is the quadratic component of the dose–effect relationship:

$$V^{\text{obs}}_o - V^{\text{obs}}_i = \alpha D + bD^2$$

$$\frac{(V^{\text{obs}}_o - V^{\text{obs}}_i)}{D} = \alpha + bD$$

The number of sodium ions released from the DNA bound state on irradiation is directly proportional to $V^{\text{obs}}_o - V^{\text{obs}}_i$ (equation 12 appendix A).

Figure 4.8 includes the quantity $V^{\text{obs}}_o - V^{\text{obs}}_i / D$ plotted against dose for comparison with the double strand break yield data divided by radiation dose. The plot of $V^{\text{obs}}_o - V^{\text{obs}}_i / D$ has zero gradient at radiation doses of less than 1 kGy. Hence linewidth narrowing does not have a quadratic dependence on dose and is thus not sensitive to DNA double strand breaks.
Figure 4.8 The difference in dose - yield relationship between i. the yield of radiation induced double strand breaks in DNA and ii. the Na-23 NMR linewidth narrowing following irradiation of DNA(aq). DNA double strand break yield data taken from [2].
4.5 Conclusions

Na-23 NMR studies indicate that a strand break induced within single stranded DNA results in the net release of 17.2 (± 2.5) sodium ions per nucleotide from the DNA bound state into bulk solution. Na-23 NMR linewidth narrowing indicates that a single strand break within double stranded DNA results in the net release of approximately 8 sodium ions per DNA base pair (i.e. 3.97 (± 0.71) sodium ions per nucleotide).

This study has shown that Na-23 NMR is a useful technique for measuring single strand breaks in both single and double stranded DNA.
Appendix A

Na-23 NMR linewidth narrowing on irradiation

Derivation of an equation describing sodium-23 NMR linewidth narrowing in terms of the number of DNA 'bound' sodium ions released per unit of absorbed radiation dose.
Appendix A

The observed Na-23 NMR linewidth $V_{\text{obs}}$ of solutions containing unirradiated DNA is given in the two state model by:

\[ NV_{\text{obs}} = n_b^i V_b + n_f^i V_f \]

where $N$ is the total number of sodium ions in solution, $n_b^i$ is the number of DNA bound sodium ions, $n_f^i$ is the number of free sodium ions in unirradiated solutions containing DNA. The sodium ion NMR linewidths in the free and bound states are $V_f$ and $V_b$ respectively. It follows from equation 2 that $NV_{\text{obs}}$ may be written in terms of $n_b^i$, equation 3.

\[ n_f^i = N - n_b^i \]

hence

\[ NV_{\text{obs}} = n_b^i (V_b - V_f) + N V_f \]

Counter-ion condensation theory yields the number of sodium ions in the DNA bound state per DNA phosphate group. Thus, $r$ is defined as the number of bound sodium ions ($n_b^i$) per DNA phosphate ($P$), equation 4.

\[ r = \frac{n_b^i}{P} \]

Introducing $r$ into equation 3 and noting that $N = P$ produces a simplified expression for the observed Na-23 NMR linewidth, equation 7.

\[ \frac{NV_{\text{obs}}}{P} = \frac{n_b^i}{P} (V_b - V_f) + \frac{NV_f}{P} \]

Thus

\[ \frac{NV_{\text{obs}}}{P} = r (V_b - V_f) + \frac{NV_f}{P} \quad \text{(N.B. } P=N) \]
therefore

\[ V_{obs}^{0} = r (V_{b} - V_{f}) + V_{f} \]

Following irradiation, sodium ions are released from the DNA bound state to the free state. Thus the number of sodium ions in the bound state \( n_b \) is less than the number of DNA bound sodium ions in unirradiated samples \( n_{b}^{*} \) and so \( n_{b} = n_{b}^{*} - \Delta n \). The observed sodium NMR linewidth \( (V'_{obs}) \) of irradiated solutions containing DNA may be expressed as:

\[ NV'_{obs} = n_{b}^{*} (V_{b} - V_{f}) - \Delta n_b (V_{b} - V_{f}) + N V_{f} \]

As above (equations 4 to 7), the number of sodium ions per DNA phosphate \( r \) may be incorporated into equation 8, along with the quantity \( \Delta r \) which is equal to \( \Delta n/P \) (equation 9).

\[ \frac{NV'_{obs}}{P} = r (V_{b} - V_{f}) - \Delta r (V_{b} - V_{f}) + \frac{NV_{f}}{P} \]

The change in the number of bound sodium ions per phosphate \( (\Delta r) \) per unit absorbed radiation dose can be defined as a parameter \( \lambda \), equal to \( \Delta r/D \). Equation 9 then simplifies to give equation 11 (N.B. \( P = N \)).

\[ \lambda = \frac{\Delta r}{D} \]

\[ V'_{obs} = r (V_{b} - V_{f}) - \lambda D (V_{b} - V_{f}) + V_{f} \]

Thus, subtracting the observed Na-23 NMR linewidth of an irradiated DNA solution \( (V'_{obs}) \) from the observed Na-23 NMR linewidth of an unirradiated \( (V^{0}_{obs}) \) sample leads to equation 12.

\[ V_{obs}^{0} - V'_{obs} = \lambda D (V_{b} - V_{f}) \]

Hence a plot of \( V_{obs}^{0} - V'_{obs} \) against dose, \( D \), has a gradient of \( \lambda (V_{b} - V_{f}) \). A plot of this type was used to determine \( \lambda \) for irradiated
solutions containing either single stranded or double stranded DNA (figures 4.5 and 4.6). These values of $\lambda$ were used to calculate the number of sodium ions released from the DNA bound state to the free state following irradiation.
4.6 References


